Received: 18 October 2023 | Revised: 15 January 2024 | Accepted: 5 March 2024

DOI: 10.1002/fft2.389

FOOD FRONTIERS Open Access

REVIEW ARTICLE

Valorization of biomass for food protein via deep eutectic solvent extraction: Understanding the extraction mechanism and impact on protein structure and properties

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Funding information
Universiti Putra Malaysia, Grant/Award Number: GP-GPB/2022/9711600

Abstract

The conventional techniques for protein extraction from biomass are not fully aligned with sustainability goals, so it is important to look for some alternate solutions. By simultaneously extracting both soluble and insoluble proteins, deep eutectic solvents (DESs) offer a viable method for valorizing protein-rich biomass from a variety of sources. Notably, the molecular crowding effects of DESs may have helped unfolded proteins acquire compact and stable conformations, facilitating solubilization and effective extraction. However, there is still a lack of information regarding how DESs interact with proteins and affect the structure and properties of the recovered proteins. To enable their widespread usage as a sustainable method for extracting dietary proteins, the safety of DES-extracted proteins must also be addressed. In this paper, we review the state of the science in DES-mediated protein extraction, focusing on the extraction mechanism and the interactions between DESs and proteins. Additionally, important aspects of DES-mediated protein extraction that could affect the structure, technofunctional, nutritional characteristics, and safety of extracted protein are explored. DES-based protein extraction could be helpful to valorize different biomasses for the production of food proteins due to the specific features.

KEYWORDS
alternative proteins, biomass valorization, deep eutectic solvent–mediated extraction, extraction mechanism, functional properties, protein structure

1 | INTRODUCTION

With a projected 10 billion people on the planet Earth in 2050, it is predicted that the world's food demand will increase by 55% (Godfray et al., 2018; Searchinger et al., 2019). In order to meet future food demands and environmental sustainability, the agri-food industry and researchers are now focusing more on the valorization of underutilized bioresources with the main goal of switching to alternative food production (Wang et al., 2021; Willett et al., 2019). The development of novel food products with fewer harmful effects on the environment and the ability to feed a growing global population has led to significant interest in proteins derived from alternative sources such as plants, microbes, and insects (Grossmann & Weiss, 2021). Alternative proteins are much more environmentally friendly than traditionally produced animal meat-based proteins because they need fewer resources (such as land and water) and thus have a lower

environmental footprint (Grossmann & Weiss, 2021; Reijnders & Soret, 2003).

Currently, research in food science and technology is focused on the investigation of alternative protein sources that offer appropriate human nutrition and have a smaller impact on the environment. These investigations cover identifying new protein sources, developing new or more effective extraction techniques, modifying technofunctional and organoleptic attributes, and devising cutting-edge processing techniques, particularly for texturization and formulation purposes (Grossmann & Weiss, 2021). Recent studies have revealed a rising market share for alternative proteins, showing a rise in customer demand and encouraging producers to use these proteins in the future (Aschemann-Witzel et al., 2021). A variety of alternative protein sources, such as legumes, grains, seeds, nuts, and leaves (Sá et al., 2020; Tenorio et al., 2018), microbial (Hashempour-Baltork et al., 2020; Sillman et al., 2019), algae (Geada et al., 2021), seaweed (Rawiwan et al., 2022), insects (Gravel & Doyen, 2020), and, more recently, cultivated meat (Jairath et al., 2021), have been explored.

The production of proteins, however, is a resource-intensive and challenging task (Reijnders & Soret, 2003). Depending on the protein source, special processing techniques may be necessary to extract the protein from the raw material before it can be used in other products. In most cases, extraction increases the protein's bioavailability and digestibility as well as its ability to fully utilize its inherent functional properties, such as its ability to gel, emulsify, and hold onto water and oil (Benhammouche et al., 2021). Proteins are first solubilized by alkaline solvents at high pH levels (>pH 8) in traditional extraction techniques, which are then followed by filtration, pH neutralization, and the recovery of the solubilized proteins (Momen et al., 2021). This process is the basis of the industrial production of soy protein and has been well documented (Day, 2013; Preece et al., 2017).

Alkaline-based protein extraction is common and straightforward, but it can also lead to a number of changes, especially at extremely high pH levels (pH > 10), such as altered secondary, tertiary, and quaternary protein structures that limit functional properties. It also leads to intensified oxidation of polyphenol compounds, resulting in the formation of dark color, and the formation of the lysinoalanine (LAL) complex that reduces protein digestibility and causes the loss of essential amino acids (Momen et al., 2021; Zhang et al., 2019). Moreover, after extraction and the subsequent acid-neutralization stage, the method also produces residual wastewater (Zhang et al., 2018). Despite being the most used method for extracting protein from foods, alkaline-mediated protein extraction has not received much attention when it comes to sustainability, with little research examining its effects on the environment.

Meanwhile, in-line with the green extraction principles coupled with growing demand from the functional food and nutra-pharmaceutical sector, there is a prompt need for the use of more environmentally friendly processes to extract high-quality protein from diversified bioresources (Willett et al., 2019). A transition toward sustainable food systems includes a move toward more efficient processing with a lower environmental footprint. Recently, there has been substantial interest in research on so-called green solvents as alternative solvents for synthesis, extraction, purification, and formulation (Vanda et al., 2018). Green solvents are a new generation of solvents that attempt to adhere to the principles of green extraction, which aim to minimize the use of toxic chemicals for various processes, while also reducing the energy consumption and providing safer and healthier end products (Clarke et al., 2018). A comprehensive review of several types of green solvents has been published elsewhere (Clarke et al., 2018; Schuur et al., 2019).

Deep eutectic solvents (DESs) have recently gained much interest because of their inherent unique properties, including simple preparation, low volatility, and high solvation power (Gullón et al., 2020). Their role as solvents for protein extraction has gained interest, but detailed information, such as the mechanism of extraction, the influence of DESs on protein structure and functional properties, bioavailability, and safety of DES-extracted proteins, remains underexplored. This review aims to provide a comprehensive overview of the most recent research on the use of DESs as solvents for food protein extraction as well as the potential impacts of DES composition and processing parameters on the yield, purity, structure, nutritional value, and nutraceutical properties of the extracted protein. In order to understand the process of DES-mediated protein extraction, it also focuses on interactions between DES and proteins.

2 | TRANSFORMING NONCONVENTIONAL AGRI-FOOD RESOURCES AND BIOMASS AS A SOURCE OF FOOD PROTEINS

The growth of the agri-food industry has led to the generation of a vast quantity of by-products, a trend that continues to expand in parallel with the rising global population. This surge in agricultural by-products has contributed to approximately 1.3 billion tons of food being lost or wasted globally each year, with the majority attributed to the fruits and vegetables category, followed by cereals, roots, and tubers (Wang et al., 2021). These by-products primarily consist of inedible portions left over after processing, yet they contain significant protein content that often goes to waste. The continual increase in the production of these by-products underscores the inefficiencies in current processing practices and industry utilization (Baker & Charlton, 2020). Therefore, upcycling waste biomass to create food proteins is consistent with the concept of a circular bioeconomy and offers great potential toward the valorization of underutilized bioresources. It not only reduces the accumulation of underutilized biomass but also addresses the challenge of managing agri-food by-products while contributing to sustainable food production (Contreras et al., 2019).

Agri-food by-products encompass a wide range of biomass, including fruit pomace, peels, oilseed meals, cereal bran, and fishery by-products, as detailed in Table 1. Such by-products represent a vast reservoir of proteins due to the sheer volume of biomass generated. For example, global potato production yields an estimated 368 million metric tons (Mt), with peels constituting 15%–40% of the whole potato (Table 1). Potato peels can contain protein content ranging from 2.10% to 17.19% (Sampaio et al., 2020), potentially providing 1.16–25.30 Mt of proteins from potato peels alone. Beyond assessing total protein

TABLE 1 Estimated global production and chemical composition of some nonconventional biomass, agri-food commodities and their by-products.

Resource	Estimated global production yield or area planted	Biomass	Percentage of biomass to total weight of resource (%)	Chemical composition (reported as dry basis unless indicated otherwise)				References
				Protein (%)	Carbohydrate (%)	Lipid (%)	Fiber (%)	
Fruits & vegetables								
Potato (*Solanum tuberosum* L.)	368 Mt	Peel	15–40	2.10–17.19	68.7–88.0	0.73–2.60	–	Sampaio et al. (2020)
Tomato (*Solanum lycopersicum*)	180 Mt	Pomace	3–5	15.08–24.67	–	2.0–16.24	39.11–68.04	Lu et al. (2019)
		Seed	1–2	23.60–40.94	–	17.80–24.50	16.00	
Pomegranate (*Punica granatum*)	3.8 Mt	Peel	50	3.26	86.52	3.31	34.0	El Barnossi et al. (2021)
Tangerine (*Citrus reticulata*)	31.7 Mt	Peel	40–50	8.55	18.03	2.97	12.8	
Grape (*Vitis vinifera* L.)	73.52 Mt	Pomace	20–25	8.49	29.20	8.16	46.17	FAOSTAT (2023), Spinei and Oroian (2021)
Pumpkin (*Cucurbitaceae*)	23.78 Mt	Peel	2.6–16	1.8–25	37–85	3.6–7.6	9.2-27	FAOSTAT (2023), Rico et al. (2020)
		Seed	3.1–4.4	21–44	9.9–70	18–55	3.4–53	
Watermelon (*Cucumis melo* L.)	101.63 Mt	Peel	30–41	7.4–18	28–85	1.1–2.6	3.1–39	
		Seed	2	18–25	13–29	24–58	2.5–49	
Pineapple (*Ananas comosus*)	28.65 Mt	Peel	29–42	0.36–9.1	14–42	1.1–2.0	16–65	
		Core	9.4–20	0.85–4.00	14	1.3–3.2	48	
Cereals								
Wheat (*Triticum aestivum* L.)	770.9 Mt	Grain	100	10.7	75.4	2.0	12.7	FAOSTAT (2023), Schmidt et al. (2021)
		Bran	15–20	15.5[a]	64.51[a]	4.25[a]	42.8[a] (DF)	Cheng et al. (2022)
		Wheat germ	2–3	28.1[a]	51.3[a]	9.6[a]	12.3[a] (DF)	Boukid et al. (2018)
Barley (*Hordeum vulgare* L.)	145.62 Mt	Grain	100	12.0	77.6	2.8	5.0	FAOSTAT (2023), Biel et al. (2020)
		Bran	10–30	16.51[a]	–	3.52[a]	14.77[a]	Farag et al. (2022), Pasha et al. (2020)
Sorghum (*Sorghum bicolor*)	61.36 Mt	Grain	100	8.1–11.1	52.2–87.14	1.65–3.38	8.52–12.86	FAOSTAT (2023), Rashwan et al. (2021)

(Continues)

TABLE 1 (Continued)

Resource	Estimated global production yield or area planted	Biomass	Percentage of biomass to total weight of resource (%)	Chemical composition (reported as dry basis unless indicated otherwise)				References
				Protein (%)	Carbohydrate (%)	Lipid (%)	Fiber (%)	
Oat (*Avena sativa* L.)	22.57 Mt	Grain	100	12–20	69–76	5–18	5–10	FAOSTAT (2023), Boukid (2021)
Amaranth (*Amaranthus* spp.)	N/A	Grain	100	13.0–17.5	60.5–67.2	6.0–7.8	2.9–5.0	Tovar-Pérez et al. (2019)
Oilseeds								
Sunflower seed (*Helianthus annuus* L)	58.19 Mt	Seed	100	20.8	–	24.9	52.18 (NDF + ADF)	FAOSTAT (2023), Ítavo et al. (2015)
Cotton seed (*Gossypium* spp.)	42 Mt	Seed	100	39.7–42.1	12.2–16.6	35–38.7	10.0	He et al. (2022)
Sesame seed (*Sesamum indicum* L.)	6.35 Mt	Seed	100	20.8	9.85	49.7	14.9	FAOSTAT (2023), Wei et al. (2022)
Hemp seed (*Cannabis sativa* L.)	0.006 Mt	Seed	100	23.8–28	–	26.9–30.6	59.1–68.3 (NDF + ADF)	FAOSTAT (2023), Vonapartis et al. (2015)
Green biomass								
Cassava (*Manihot esculenta*)	314.81 Mt	Leaves	–	19.7–38.1	31.9–64.7	3.5–7.3	8.3–19.5	FAOSTAT (2023), Parmar et al. (2017)
Sugar beet (*Beta vulgaris* L.)	270.16 Mt	Pressed pulp	–	10.42	82.64	0.84	75.20 (TDF)	FAOSTAT (2023), Pacheco et al. (2019)
		Leaves	20–34	14.8	–	2.9	30.3 (TDF)	Tenorio (2017)
Moringa oleifera	43–115 t/ha/year	Leaves	–	22.42	27.05	4.96	30.97 (DF)	Su and Chen (2020), Sánchez-Machado et al. (2010)
		Seed	–	31.4	18.4	36.7	7.3	Leone et al. (2016)
Spinach (*Spinacia oleracea* L.)	32.29 Mt	Leaves	100	27.8 30.42	30.28 33.13	5.69 6.22	8.82 9.65	FAOSTAT (2023), Galla et al. (2017)

(Continues)

TABLE 1 (Continued)

Resource	Estimated global production yield or area planted	Biomass	Percentage of biomass to total weight of resource (%)	Chemical composition (reported as dry basis unless indicated otherwise)				References
				Protein (%)	Carbohydrate (%)	Lipid (%)	Fiber (%)	
Green biomass								
Broccoli (*Brassica oleracea* L. var. Italica)	~27 Mt	Leaves	47	21.26	–	3.34	62.22 (TDF)	Rivas et al. (2022), Liu et al. (2018)
Alfalfa (*Medicago sativa* L.)	30 million ha	Sprouts	100	22.44[a]	61.84[a]	3.35[a]	33.34[a] (TDF)	Acharya et al. (2020), Djordjević et al. (2023)
Duckweed (Lemnaceae)	39–105 t/ha/year	Whole biomass	100	16.0–41.7	17.6–35.0	3.4–9.0	8.8–29.7	Zhou et al. (2023), Xu et al. (2023)
Animal-based								
Fisheries by-products	~20 Mt	Head	21.5	57.9	–	19.2	1.2	Nawaz et al. (2020)
		Viscera (gut, liver, roe, lungs)	21.8					
		Backbone	15.3					
		Skin	3.3					
		Fins & lungs	6.1					
		Skinned fillet	36.9					
Atlantic salmon (*Salmo salar*)	2.36 Mt	Heads	–	14.2	–	26.9	–	Tacon (2019), Aspevik et al. (2021)
		Viscera	–	9.9	–	34.5	–	
Gilthead sea bream (*Sparus aurata*)	N/A	Head	16.07–18.49	32.40–37.19	12.41–12.66	28.76–37.08	–	Kandyliari et al. (2020)
		Intestines	4.49–5.44	26.87–37.23	15.39–16.01	43.19–55.12	–	
		Skin	6.22–6.35	43.16–49.67	0.03–4.43	45.94–46.39	–	
Sardine (*Sardina pilchardus*)	N/A	Viscera	–	15.76[a]	–	4.89[a]	–	Kechaou et al. (2009)
Chicken feathers	~9.5 Mt	–	–	84.28	–	8.08	–	Pedrosa et al. (2022)

Abbreviations: ADF, acid detergent fiber; DF, dietary fiber; Mt, million metric tons; NDF, neutral detergent fiber; t, tons; TDF, total dietary fiber.
[a] Reported as fresh weight basis or unspecified.

content, we must also consider the nutritional quality of these biomass proteins, including the presence of essential amino acids.

Efficient extraction and processing methods can transform these materials into valuable sources of food proteins suitable for various food applications. This transformation of waste biomass into valuable food proteins offers significant potential for addressing both environmental and nutritional challenges. Nonconventional protein sources present a sustainable solution for enhancing the global protein supply chain, complementing conventional sources. Although plant-based biomass has gained attention due to the growing awareness of the importance of shifting toward plant-based foods, animal-derived biomass should not be overlooked. For example, Table 1 shows that approximately 9.5 Mt of chicken feathers are generated globally, containing around 85% protein. If successfully upcycled, chicken feathers could become a valuable source of protein or amino acids. The high protein production potential of diverse bioresources offers opportunities for innovation in food science and technology.

3 | DEEP EUTECTIC SOLVENTS (DESS) AS GREEN EXTRACTION MEDIA

DESs are liquid mixtures created by combining specific hydrogen bonding donors (HBDs) and hydrogen bonding acceptors (HBAs) in precise ratios. This combination results in a mixture with a significantly lower melting temperature compared to its individual components (Kist et al., 2017; Paiva et al., 2014). The depression in melting temperature is brought about by interference of the ability of the precursor compounds to crystallize, by the formation of hydrogen bonds between the precursor compounds (Li & Row, 2016). Abbott et al. (2003) demonstrated that the combination of choline chloride (ChCl) and urea in a 1:2 molar ratio, resulted in a mixture with a melting point of 12°C, compared to 302 and 133°C for ChCl and urea, respectively. DESs are considered green solvents with potential for industrial applications, although a few researchers have shown concerns about their complete adherence to green principles (Chen & Mu, 2021; Zaib et al., 2022). The "greenness" of these solvents can be described by a set of 12 principles aiming to adhere to the concept of green chemistry (Anastas & Eghbali, 2010). Azzouz and Hayyan (2023) noted that not all DESs could be described as green solvents because of varying toxicity profiles and biodegradability rates observed for different HBA:HBD combinations. Nevertheless, researchers continue to work on improving the greenness of DESs due to their potential as environmentally friendly alternatives (Chen & Mu, 2021). DESs are often discussed together with ionic liquids (ILs), as they share common features, including low volatility, ability to dissolve a wide variety of substances, and tunable properties by virtue of varying their composition ratio (Dai et al., 2013). Tuning the composition of DESs will also alter their physicochemical properties (i.e., polarity, viscosity, pH, and density) that govern their overall performance. The physicochemical properties of some DESs are presented in Table 2. DESs are distinct from ILs, and among the major differences between these two solvents are that DESs preparation is simpler, lower cost, and excessive toxicity of ILs compared to DESs (Płotka-Wasylka et al., 2020). DES synthesis occurs as a 100% total conversion of the precursors into the final product without any by-products, which eliminates any purification or refining steps (Hansen et al., 2021; Krishnan et al., 2020).

DESs can be formed using a vast selection of materials, as documented by Dai et al. (2013). As the composition of DES changes, so do its properties, leading to its utilization in various applications, extensively discussed in several publications (Cheng & Qi, 2021; Florindo et al., 2019; Kalhor & Ghandi, 2019; Smith et al., 2014; Zhang et al., 2012). Of particular interest is its use in biomass processing, where researchers were able to utilize DESs to valorize lignocellulosic biomass and enhance saccharification, both of which play a major role in biorefinery schemes as part of the circular economy mandate to reduce waste and transform waste into value-added products (Sherwood, 2020). Biorefineries are analogous to petrochemical refineries, providing a platform for the production of various chemicals (O'Callaghan, 2016). In a future scenario where food supply chains may become disrupted due to climate change, a circular economy approach fits well with sustainability initiatives and helps optimize resource utilization. DESs made from precursor materials that are renewable, biodegradable, and biocompatible are often identified as natural DESs (NADES), whereby precursor materials can be sustainably derived from various biorefinery pathways (Atilhan & Aparicio, 2021; Clarke et al., 2018).

Previously, biomass valorization was more oriented toward the conversion or recovery of carbohydrates, fermentable sugars, and lignin, whereas the fractions containing amino acids and proteins were relatively ignored (De Schouwer et al., 2019; Scopel & Rezende, 2021). The biorefinery concept has since seen increased interest in integrating protein extraction into the overall process, increasing the potential output of a particular biorefinery and maximizing biomass utilization (Alonso et al., 2017; Contreras et al., 2019; Torres et al., 2022). This might encourage the use of DESs as solvents for protein extraction, but before any modifications can be made, it might be necessary to do additional techno-economic analyses.

4 | CONVENTIONAL FOOD PROTEIN EXTRACTION AND PROTEIN SOLUBILITY

Proteins extracted from biomass are typically entangled within the raw material, consisting of lipids and fibers, as well as minor constituents, such as phenolic compounds and minerals (Day et al., 2022). Animal-derived proteins are an exception. Food protein extraction methods are broadly divided into dry and wet fractionation methods, which focus on separating the protein fraction contained within the biomass or sample matrix (Allotey et al., 2022) (Figure 1). Dry fractionation utilizes differences in particle size and density or charge potential affected by an external electrical field after dry milling to separate the particles by air classification or electrostatic fractionation (Assatory et al., 2019). Overall, protein concentrates produced using this method possess good functional properties owing to the absence of extreme processing conditions that may cause denaturation and therefore degradation

TABLE 2 Physicochemical properties of some commonly used deep eutectic solvents (DESs).

DES (HBA–HBD)	Molar ratio (HBA:HBD)	pH	[a]Polarity, E_{NR} (kcal mol^{-1})	Viscosity (mPa s, 30°C)	Reference
Water	–	–	48.20	–	Gabriele et al. (2019)
ChCl–urea	1:1	4.1	47.26	–	Jurić et al. (2021)[b]
	1:2	10.97	–	–	Sirvio et al. (2022)
	1:2	10.13	–	449	Xu et al. (2019)[c]
ChCl–glycerol	1:1	5.2	46.87	–	Jurić et al. (2021)[b]
	1:1	–	50.91	–	Huang et al. (2017)
	1:2	4.8	–	188	Xu et al. (2019)[c]
	1:2	–	50.0	–	Oh et al. (2020)
ChCl–ethylene glycol	1:1	–	47.4	–	Mulia et al. (2019)
	1:2	–	47.4	–	Mulia et al. (2019)
	1:2	6.49	–	35	Xu et al. (2019)[c]
ChCl–acetic acid	1:2	–	48.5	–	Oh et al. (2020)
ChCl–lactic acid	1:1	1.8	44.88	–	Jurić et al. (2021)[b]
	1:1	1.29	–	508.17	Xu et al. (2019)[c]
	1:2	–	48.3	–	Oh et al. (2020)

Note: HBA:HBD refers to hydrogen bond acceptor:hydrogen bond donor.
Abbreviations: ChCl, choline chloride; HBA, hydrogen bonding acceptor; HBD, hydrogen bonding donor.
[a]Polarity as measured by Nile red as a solvatochromic probe. Solvents with higher polarity have lower E_{NR} values (Jurić et al., 2021).
[b]pH was measured in an water-diluted DES at 0.5 mol dm^{-3} (v/v).
[c]pH was measured in aqueous 75% DES mixture.

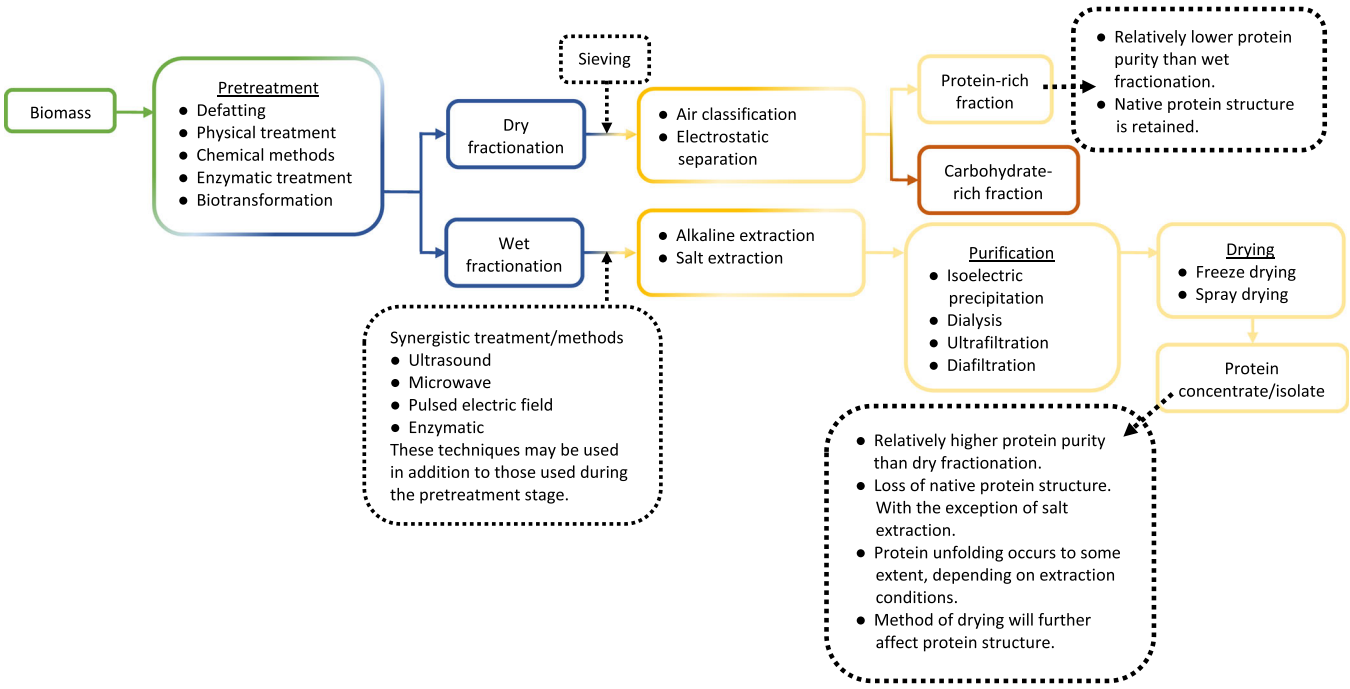

FIGURE 1 An overview of available processes for protein extraction from biomass.

in functionality (Pelgrom et al., 2013). However, dry fractionation is limited to producing protein concentrates with lower purity, which limits its use in certain applications (Allotey et al., 2022). Wet fractionation methods, on the other hand, are necessary to yield protein isolates with a high protein content, typically >90% (Tabtabaei et al., 2016). Wet extraction of food proteins relies on the solubilization of the protein with a solvent, which influences the diffusion of soluble proteins into the solvent phase. The solubilized proteins were then centrifuged to

separate them from the insoluble fraction, which contained other constituents of the raw material. This will be followed by a concentration procedure, either by isoelectric precipitation or ultrafiltration, further purifying the protein fraction. Finally, the concentrated protein fraction will be dried by spray drying or freeze drying, where differences in drying methods can affect the quality of the final product (Brishti et al., 2020; Özdemir et al., 2022).

In wet fractionation methods, water is typically the solvent used for food protein extraction; thus, protein–water interactions play a major role in determining the overall separation and recovery (Sathe et al., 2018). Protein solubility can be viewed as a balance between the attractive and repulsive molecular interactions of protein–water and protein–protein interactions. Understanding the interplay and balance between protein solubility and insolubility is the key toward designing suitable extraction processes (Grossmann & McClements, 2023). Proteins are also sensitive to processing conditions and the nature of the extraction solvent that may affect their solubility, which depends upon parameters such as pH, ionic strength, and temperature (Sathe et al., 2018).

The choice of solvent and processing conditions play a critical role, as they affect the extraction yields and functional properties of extracted proteins (Cabral et al., 2022; Corredig et al., 2020). Extraction can be complicated because proteins vary greatly among different sources in terms of size, conformation, and amino acid composition. For example, plant proteins can be classified as albumins, globulins, glutelins, and prolamins, which are soluble in water, salt, acid/alkali, and alcohol, respectively. This was specified by Osborne (1908), who characterized protein solubility for various plant proteins, and this nomenclature has since been used to categorize proteins from different sources such as seaweed (Mendez & Kwon, 2021) and insects (Caligiani et al., 2018). Such categorization allowed us to compare among proteins from different sources and highlight differences among them. For example, legumes and cereals contain various proteins with different solubility profiles. Legumes are abundant in albumins and globulins, whereas cereals contain more prolamins and glutelins that are less soluble in aqueous solutions (Loveday, 2019). Möller et al. (2022) employed a mild wet fractionation process that takes into account the difference between the isoelectric point (p*I*) of yellow pea albumins and globulins, enabling the recovery of both types of proteins.

Furthermore, the location and nature of proteins play a crucial role in their extractability. Proteins are distributed across various cellular compartments, and their properties, including structure, conformation, and physicochemical attributes, vary depending on their specific location within the biomass. Consequently, diverse extraction techniques are required to accommodate the variability in protein structure and location. That variability also extends toward the overall nature of the protein itself, which can form complexes with other macromolecules (e.g., glycoproteins and lipoproteins) that influence its structure, function, and properties and therefore affect their extractability. Industrial-scale production of protein isolates and concentrates often involves extracting soluble storage proteins found in legumes like soybean and pea. These proteins accumulate in protein bodies, serving the plant during maturation (Shewry et al., 1995).

Dry or wet fractionation methods effectively separate these proteins from other cellular components. Conventional extraction methods typically yield protein concentrates/isolates (e.g., soy) at approximately 50%–70%, leaving a significant portion of proteins potentially unused (Tenorio et al., 2018). Unextracted proteins remaining in the biomass can consist of soluble proteins entrapped within the biomass matrix, insoluble aggregated proteins with high molecular weights, or integral proteins embedded within membranes (Abdullah et al., 2020; Rommi et al., 2014). Meanwhile, animal-based by-product biomass (i.e., animal parts and feathers) contains structural proteins such as collagen and keratin. However, extracting these proteins can be difficult due to their fibrous polymeric composition, which is highly resistant to degradation and solubilization (Ghaffari-Bohlouli et al., 2022). Suboptimal extraction procedures requiring long extraction times and potential use of hazardous chemicals to facilitate solubilization jeopardize the long-term feasibility and sustainability of such processes (Ghaffari-Bohlouli et al., 2022). Understanding the nature of proteins from different sources and their solubility allows us to design processes and select suitable solvents for maximal extraction efficiency.

5 | BARRIERS TOWARD SOLUBILIZATION OF BIOMASS PROTEIN

Proteins from various sources are typically located within cellular compartments, which require disruption before they can fully interact with solvents, especially with plant-based sources (Kumar, Tomar, et al., 2021; Ursu et al., 2014). There are several methods available at our disposal to disrupt cellular barriers to enhance protein solubilization. These methods may involve enzymatic, physical, biotransformation, and chemical treatment by using different solvents, which can further aid protein release into the extracellular space, or a combination of methods, most commonly employed as pretreatment prior to protein extraction (Tu & Hallett, 2019). In general, the chemical compositions of biomass are composed of varying degrees of proteins, lipids, and ash, whereas plant-based biomass contains more carbohydrate fractions such as cellulose and hemicellulose, and lignin. These are the major components considered during the processing of such raw materials. Protein extraction from biomass depends, to some degree, on the biomass composition. Sari et al. (2015) employed an alkaline aqueous solution to study the extractability of proteins from various biomasses and demonstrated that protein extraction was affected to a certain extent by the presence of cellulose and lipid content of biomass.

Lipid removal often precedes protein extraction in biomass processing as a matter of practicality and necessity. The presence of lipids interferes with protein extraction due to the ability of proteins to adsorb onto lipids, forming emulsions that could reduce extraction yields (Laroche et al., 2019). Organic solvents such as hexane are traditionally employed in lipid extraction to maximize oil yield and defatting

purposes prior to protein extraction, but these solvents are highly volatile and hazardous from an environmental and health perspective (Nde & Anuanwen, 2020). Consequently, conventional screw-pressing, nonconventional, and sustainable processes have been used or developed to minimize the use of organic solvents. Aqueous extraction techniques allow for the co-extraction of lipids and proteins, and this has been extensively documented (Chen, Wang, et al., 2019; de Souza et al., 2020; Liu et al., 2016). These methodologies result in the lipid fraction being accumulated in an oil-rich cream layer, partly stabilized by the proteins. The protein-rich skim fraction also contains significant amounts of lipids (~10%), probably caused by interactions between lipids and exposed hydrophobic regions of proteins (Almeida et al., 2021). Suitable strategies need to be developed to produce protein isolates from skim fractions of oil- and protein-co-extracted raw materials.

The vast majority of plant biomass cell walls are predominantly composed of cellulose, hemicellulose, and lignin, collectively termed lignocellulose (Akhtar et al., 2015). These structural components often present a barrier toward the extraction of various biomolecules, thus requiring pretreatment strategies to disrupt the structure (Woiciechowski et al., 2020). Plant cell wall structure is typically organized in a particular way, as depicted in Figure 2a–c. Cellulose is present in fibrous bundles, interwoven together with hemicellulose and lignin, which provides rigidity and recalcitrance toward solvents and enzymes (Kumar, Bhardwaj, et al., 2021). Cellulose is a polymer composed of β-1,4 linked D-glucose units, whereas hemicellulose is a mix of polysaccharides of different carbohydrate monomers, which varies from biomass to biomass (Zhou et al., 2017). Due to their molecular characteristics, cellulose polymer chains can be associated in close packing configuration, forming crystalline-like complexes. Together, they present a matrix that is highly resistant to depolymerization (Zoghlami & Paës, 2019). As a consequence, these complexes are not easily soluble in aqueous solutions, and obtaining cellular proteins can be a challenging task.

The impermeable nature of the polysaccharide barrier often necessitates the pretreatment of biomass to improve their digestibility to enzymes or accessibility to solvents. Various pretreatment techniques have been described in a number of papers that use chemical, enzymatic, or physical methods to break down cellular barriers and improve biomolecule solubilization (Haldar & Purkait, 2021; Kalhor & Ghandi, 2019; Khan et al., 2022). Given that some DESs have been demonstrated to be capable of dissolving crystalline cellulose complexes and lignin, the use of DESs in the processing of biomass is an innovative use (Hassan & Mutelet, 2022). By dissolving the polysaccharide barrier using DESs, intracellular proteins may be released for later recovery, giving biorefinery designs an additional choice for effectively using biomass. The capacity of DESs to saturate proteins, including water-insoluble ones, is another noteworthy feature (Wahlström et al., 2017). Considering the growing threat posed by climate change, the ability of DESs to solubilize water-insoluble proteins opens up new opportunities for the production of food proteins from various bioresources.

6 | DES UTILIZATION IN PROTEIN EXTRACTION

Protein extraction may be difficult due to the biomass matrix's complex chemical makeup. Protein extraction efficiency can vary significantly between different processing methods (Contreras et al., 2019). As a more convenient and ecologically friendly alternative to traditional alkaline-based extraction techniques, DES-mediated protein extraction has gained popularity. Figure 3 shows two different procedures for DES-mediated protein extraction from biomass. With these methods serving as a guide, researchers have added steps or adjusted the conditions depending on their requirements. Moreover, a summary of protein extraction processes utilizing DESs together with their efficacy is presented in Table 3.

6.1 | Extraction of protein from plant biomass using DESs

There are huge resources of underutilized agro- and fruit-processing wastes that can be explored as viable biomass feedstocks for the extraction of food proteins. For example, brewer's spent grain (BSG) is a by-product of the brewing industry; its production is estimated to be approximately 37.2 million tonnes worldwide in 2021, illustrating its abundance and potential as a biorefinery feedstock (Agrawal et al., 2023). BSG is lignocellulosic by nature; its cellulose, hemicellulose, and lignin content as a collective make up the majority of its composition, whereas proteins (14.5%–30%) are the next largest component of BSG (Naibaho & Korzeniowska, 2021; Qazanfarzadeh et al., 2023). A variety of methods, including chemical, physical, or a combination of those techniques, have been used to extract BSG proteins, with varying yields and functional properties (Connolly et al., 2013; Niemi et al., 2013; Li, Yang, et al., 2021).

As demonstrated by previous research, the extraction of BSG proteins by conventional solvents (typically NaOH-based aqueous solutions) is limited by the fact that the majority of BSG proteins are composed of hordeins, a prolamin-type storage protein most soluble in alcohol (Jaeger et al., 2021; Wen et al., 2019). Alcohols are not used extensively as protein extraction solvents but are commonly used for the extraction of plant secondary metabolites and other volatile components (Chemat et al., 2019). Wahlström et al. (2017) showed that a sodium acetate–urea (1:2) DES containing 10% water was able to extract protein from BSG with up to 79% yield. Epifluorescence microscopy showed that sodium acetate–urea (1:2) was able to extract BSG proteins that are typically present in aggregated form, leading to higher extraction yield.

Grudniewska et al. (2018) used ChCl–glycerol (1:2) to extract proteins from rapeseed cake and evening primrose cake, varying the temperature to observe the impact of temperature upon protein extraction. They reported that increased precipitate yields were obtained after DES treatment with increasing temperature, for both sample types. SDS–PAGE analysis appears to confirm the presence of cruciferin bands in DES-treated rapeseed cake precipitates, which indi-

TABLE 3 Effect of deep eutectic solvent (DES)-mediated protein extraction variables on yield and purity of protein isolates from various biomasses.

Protein source	DES (molar ratio of HBA to HBD)	Extraction parameters	Extraction yield	Protein yield	Protein content ($N \times 6.25$)	Reference
Plant biomass						
Brewer's spent grain	Sodium acetate–urea (1:2), with 10% water (wt%)	Temperature: 80°C Time: 2 h Solid/Solvent ratio: 1:10 (w/w)	79%	–	54.7%	Wahlström et al. (2017)
	ChCl–urea (1:2)	Temperature: 80°C Time: 2 h Solid/Solvent ratio: 1:20 (w/w)	23%	–	52%	
Flour	ChCl–ethylene glycol (1:2)	Temperature: 55°C Time: 45 min Solid/Solvent ratio: 1:10 (w/v)	–	94%	–	Svigelj et al. (2017)
Rapeseed cake	ChCl–glycerol (1:2)	Temperature: 60–140°C Time: 2 h Solid/Solvent ratio: 1:9 (w/w)	11.5%–19.9%	–	36%–48%	Grudniewska et al. (2018)
Evening primrose cake (*Oenothera biennis* L.)	ChCl–glycerol (1:2)	Temperature: 60–140°C Time: 2 h Solid/Solvent ratio: 1:9 (w/w)	8.4%–34.2%	–	40%–50%	
Bamboo shoot tip (*Bambusa oldhamii*)	ChCl–levulinic acid (1:6)	Temperature: 80°C Time: 50 min Solid/Solvent ratio: 30 mg/mL Water content: 40% (v/v)	39.16 mg/g dry wt bamboo tip	–	–	Lin et al. (2021)
Orange peel	ChCl–ethylene glycol (1:2), with 50% water (wt%)	Solid/Solvent ratio: 1:4 (w/v)	7.7 mg/g peel	–	–	Panić et al. (2021)
Soybean (*Glycine max*)	ChCl–glycerol (1:3)	Temperature: 60°C Time: 3.9 h Solid/Solvent ratio: 10:103 (w/v) Stirring: 873 rpm	0.3462 g/g soybean	–	–	Chen et al. (2021)
Melon peel (*Cucumis melo* L.)	Sodium acetate–urea–H_2O (1:3:1.6)	Temperature: 90°C Time: 1 h Solid/Solvent ratio: 1:50 (w/v)	39.91%	–	–	Rico et al. (2021)
Oat	ChCl–1,4-butanediol–H_2O (1:3:1)	Temperature: 80°C Time: 90 min Solid/Solvent ratio: 1:9 (w/w)	7.98%	31.09%	55.28%	Yue et al. (2021)
	ChCl–1,3-propanediol–H_2O (1:3:1)	Temperature: 80°C Time: 90 min Solid/Solvent ratio: 1:9 (w/w)	6.34%	25.01%	56.38%	Yue et al. (2022)
Plant biomass						
Seabuckthorn seed meal (*Hippophae rhamnoides* L.)	ChCl–urea (1:2)	Temperature: 60°C Time: 3 h Solid/Solvent ratio: 1:9 (w/w) Agitation: 250 rpm	–	32.3%	67.5%	Lin et al. (2022)
Spent hops (*Humulus lupulus* L.)	ChCl–ethylene glycol (1:2), with 10% water (wt%)	Temperature: 60°C Time: 1 h Solid/Solvent ratio: 1:50 (w/w)	–	–	64%	Grudniewska & Pastyrczyk (2022)

(Continues)

TABLE 3 (Continued)

Protein source	DES (molar ratio of HBA to HBD)	Extraction parameters	Extraction yield	Protein yield	Protein content ($N \times 6.25$)	Reference
Camelina seed cake (*Camelina sativa*)	ChCl–glycerol (1:2)	Temperature: 90°C Time: 2 h Solid/Solvent ratio: 1:9 (w/w) Agitation: 550 rpm	~35%	–	~50%	Parodi et al. (2023)
Flaxseed cake (*Linum usitatissimum*)	ChCl–glycerol (1:2)	Temperature: 90°C Time: 2 h Solid/Solvent ratio: 1:9 (w/w) Agitation: 550 rpm	~34%	–	~78%	
Sunflower seed cake (*Helianthus annuus*)	ChCl–glycerol (1:2)	Temperature: 90°C Time: 2 h Solid/Solvent ratio: 1:9 (w/w) Agitation: 550 rpm	~7%	–	~58%	
Sacha inchi seed meal (*Plukenetia volubilis* L.)	ChCl–glycerol (1:2)	Temperature: 90°C Time: 3 h Solid/Solvent ratio: 1:9 (w/w) Stirring: 250 rpm	~26%	–	58.32%	Sharma et al. (2023)
Fava bean (*Vicia faba* L.)	ChCl–glycerol (1:2), with 40% water (wt%)	Temperature: 50°C Time: 1 h Solid/Solvent ratio: 1:28 (w/w) Agitation: 200 rpm	124.48 mg/g fava bean	65.42%	92.33%	Hewage et al. (2024)
Animal biomass						
Wool	ChCl–urea (1:2)	Temperature: 130°C Time: 5 h Solid/Solvent ratio: 1:100 (w/w)	35.1 mg/g	–	–	Jiang et al. (2018)
	ChCl–oxalic acid (1:2)	Temperature: 110°C Time: 2 h Solid/Solvent ratio: 5% (w/w)	~74%	–	–	Wang and Tang (2018)
	L-Cysteine–lactic acid (2:20 w/v)	Temperature: 95°C Time: 3.5 h Solid/Solvent ratio: 0.4:20 (w/v)	90%	–	–	Shavandi et al. (2021)
	L-Cysteine–lactic acid (1.6:20 w/v)	Temperature: 105°C Time: 8 h Solid/Solvent ratio: 0.5:20 (w/v)	93.77%	–	–	Okoro et al. (2022)
	Urea–lactic acid (1:2)	Temperature: 4°C Time: 48 h Solid/Solvent ratio: 1:10 (w/v)	5.2%	–	–	Bisht et al. (2021)
Blue shark skin (*Prionace glauca*)	citric acid–xylitol–H_2O (1:1:10)	Temperature: 40°C Time: 1 h Solid/Solvent ratio: 1:10 (w/v)	18.6%	16.1%	86.5%[a]	Batista et al. (2022)
Sardine heads and offal (*Sardina pilchardus*)	Betaine–propylene glycol (1:3)	Temperature: 80°C Time: 18 h Solid/Solvent ratio: 1:80 (w/w) Stirring: 60 rpm	~160 mg/g dry residue	–	–	Rodrigues et al. (2021)
Chicken feathers	Sodium acetate–urea (1:2), with 10% water (wt%)	Temperature: 100°C Time: 6 h Solid/Solvent ratio: 1:49 (w/w)	86%	45%	–	Nuutinen et al. (2019)

(Continues)

TABLE 3 (Continued)

Protein source	DES (molar ratio of HBA to HBD)	Extraction parameters	Extraction yield	Protein yield	Protein content ($N \times 6.25$)	Reference
	ChCl–phosphoric acid (1:5)	Temperature: 110°C Time: 6 h Solid/Solvent ratio: 0.2:25 (w/w)	82.02%	–	–	Zhang et al. (2023)
	ChCl–phosphoric acid–$ZnCl_2$ (1:5:3)	Temperature: 110°C Time: 6 h Solid/Solvent ratio: 0.2:25 (w/w)	85.46%	–	–	

Abbreviations: ChCl, choline chloride; HBA, hydrogen bonding acceptor; HBD, hydrogen bonding donor.
[a]Measured using Bradford assay.

cates that ChCl–glycerol (1:2) could be selective toward the extraction of cruciferin rather than napin proteins (Grudniewska et al., 2018). However, research from Li et al. (2016) and Meng et al. (2019) suggested that DESs are nonselective toward proteins, and that different DESs possess different extraction efficacies. Depending on the DES constituents, some may extract proteins efficiently, whereas others do not. Therefore, screening of DESs for protein extraction efficiency is important to select the best performing DESs. Rapeseed is an important oilseed crop, generating rapeseed cake as a by-product after oil extraction, containing 37%–41.4% protein (Chmielewska et al., 2020). Due to the presence and concurrent extraction of antinutritional components like glucosinolates and phytate as well as a polysaccharide matrix that traps and prevents rapeseed protein from being solubilized, the extraction of rapeseed proteins is regarded as a difficult process (Chmielewska et al., 2020; Rommi et al., 2014). The precipitates' purity, which contained 36%–48% protein, reflects this. The given protein recovery procedure differs from that used by Wahlström et al. (2017), who centrifuged the original extract before re-extracting the pellet with DES three more times, a step that would have increased the nonprotein content.

The approach of Grudniewska et al. (2018) was also employed in research by Parodi et al. (2023) to extract proteins from flax, camelina, and sunflower oilseed press cakes. Elemental analysis after precipitation with water showed that the precipitates' nitrogen content was larger than that of the corresponding oilseed meals, and that this nitrogen content was further increased by raising the extraction temperature from 60 to 90°C. This agrees with the findings presented by Grudniewska et al. (2018). ATR-FTIR analysis appeared to confirm their claim that the precipitates contain proteins, as shown by their findings that the precipitates exhibit stronger amide I and II band absorption than the remaining press cakes (Parodi et al., 2023).

The yields attained by Parodi et al. (2023) did not, however, match those obtained by Wahlström et al. (2017) and were comparable to those achieved by Grudniewska et al. (2018). Given that each raw material was subjected to varying degrees of pretreatment, variability in the raw materials may have played a role. In this instance, using 90°C led to the highest yields. When the temperature was raised from 100 to 130°C, Hassan and Mutelet (2022) demonstrated that *Miscanthus*, a perennial grass high in lignocellulose, had improved solubility by ChCl–glycerol (1:2) pretreatment. Thus, it can be assumed that DESs may disrupt the lignocellulosic matrix more readily at high temperatures, which might result in higher protein extraction yields. However, these proteins would then cluster and form large molecular weight complexes, which would be captured by the biomass matrix, reducing the extraction yield.

The extraction of proteins from sea buckthorn seed meal using ChCl–oxalic acid, ChCl–urea, and ChCl–glycerol was compared to alkaline extraction in a study by Lin et al. (2022). In terms of extraction and recovery, they combined the techniques proposed by Wahlstrom et al. (2017), Bai et al. (2017), and Grudniewska et al. (2018). Among the DESs tested, ChCl–glycerol had the highest protein recovery, which was 41.4%, followed by ChCl–urea (32.3%) and ChCl–oxalic acid (31%). DES extraction recorded lower recovery (31%–41%) when compared to alkaline extraction at pH 11 (56.9%), along with slightly lower protein content (64.3%–67.5% against 73.1%). However, they did not optimize DES extraction parameters in the study, which could be the reason of the lower DES-extracted protein recovery yields. Notably, the raw material was pretreated with supercritical CO_2 (SC-CO_2). SC-CO_2 has been shown to cause protein denaturation and aggregation, as demonstrated by Ding et al. (2020) and Zhao et al. (2016). The severity of the SC-CO_2 process was not clear, but it is possible that SC-CO_2 may have caused sea buckthorn seed meal proteins to become aggregated, thus becoming trapped within the biomass matrix. Moreover, as described in their protocol, the DES–biomass mixture was centrifuged before the DES fraction was separated. Because most DESs are more viscous than aqueous solvents, it is likely that proteins that DESs solubilized after centrifugation were left in the biomass fraction, lowering extraction yields. Therefore, it can be emphasized that in order to evaluate the effectiveness of DES-mediated protein extraction, it is primarily important to ascertain how pretreatment affects the overall yield of protein extraction.

Soybeans, which make up the majority of plant-based protein sources, account for almost two thirds of total protein meal production globally (Organisation for Economic Co-operation and Development (OECD)/Food and Agriculture Organization (FAO), 2023). The future availability of protein in our food supply could be significantly impacted

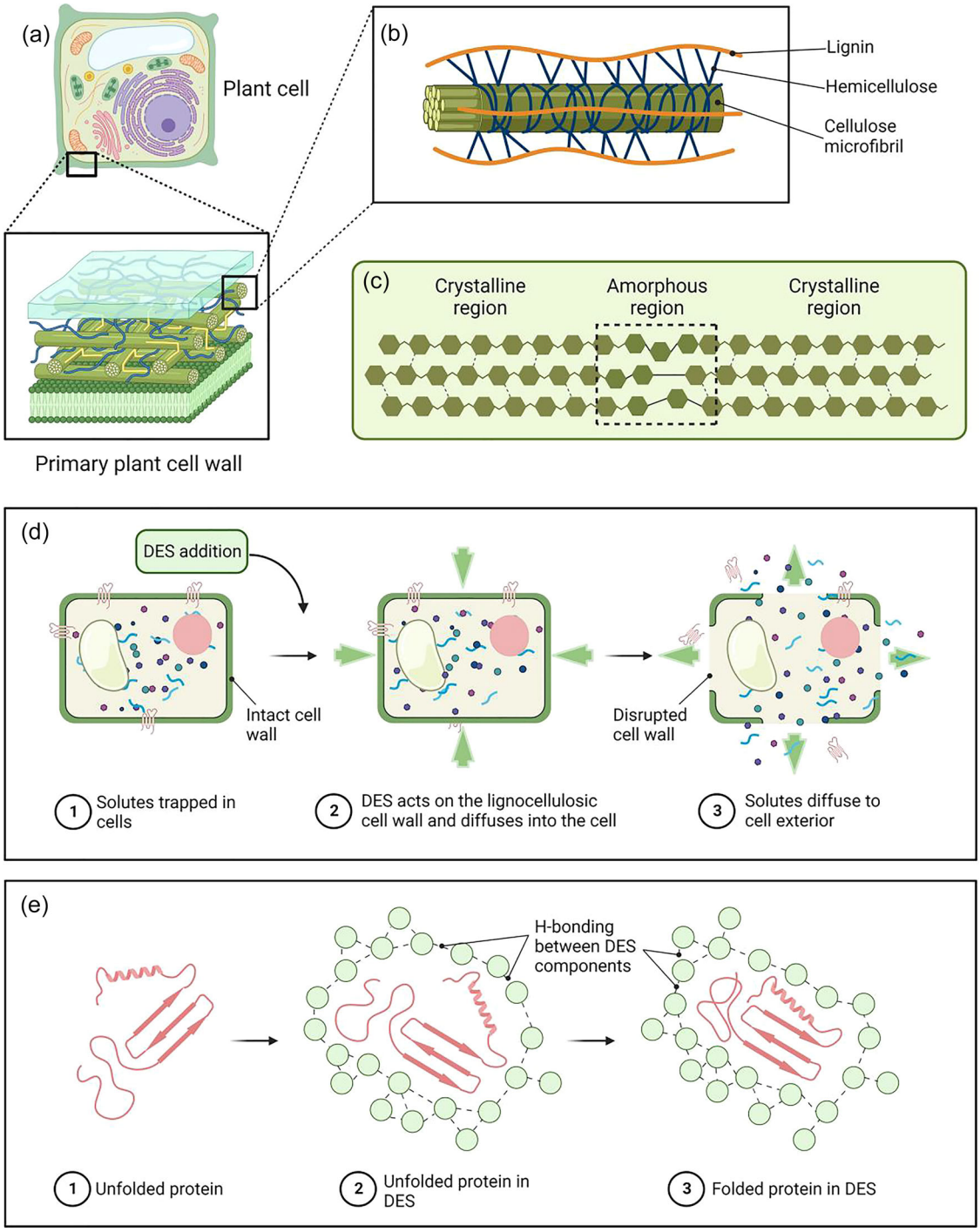

FIGURE 2 Generalized illustrations for mechanism of deep eutectic solvent (DES) extraction and interaction from plant biomass: (a) a typical plant cell; (b) the major structural components present in lignocellulosic materials; (c) the distinction between crystalline and amorphous regions within cellulose microfibers; (d) the proposed mechanism for DES-mediated protein solubilization from lignocellulosic biomass and diffusion into extracellular space. As DES is added, it begins to disrupt the cell wall and diffuses into the cell interior, thus solubilizing proteins and facilitating protein diffusion toward cell exterior; (e) the proposed sequence for DES acting as a "crowder," forcing unfolded protein to adopt a compact configuration, thus increasing solubility and stabilizing the protein against aggregation.

by innovations that improve the efficiency of protein extraction from this important resource. Chen et al. (2021) used ChCl-based DES in conjunction with various polyols as HBD to study the extraction of soy proteins. The examined HBDs were performed in the following order: 1,4-Butanediol came first, followed by 1,2-butanediol, glycerol, ethylene glycol, and polyethylene glycol. They then employed ChCl–glycerol (1:3) and the response surface approach to optimize the stirring speed, duration, and liquid/solid ratio in order to obtain the optimal set of parameters for soybean protein extraction. The amount of protein recovered from each gram of soybean using the

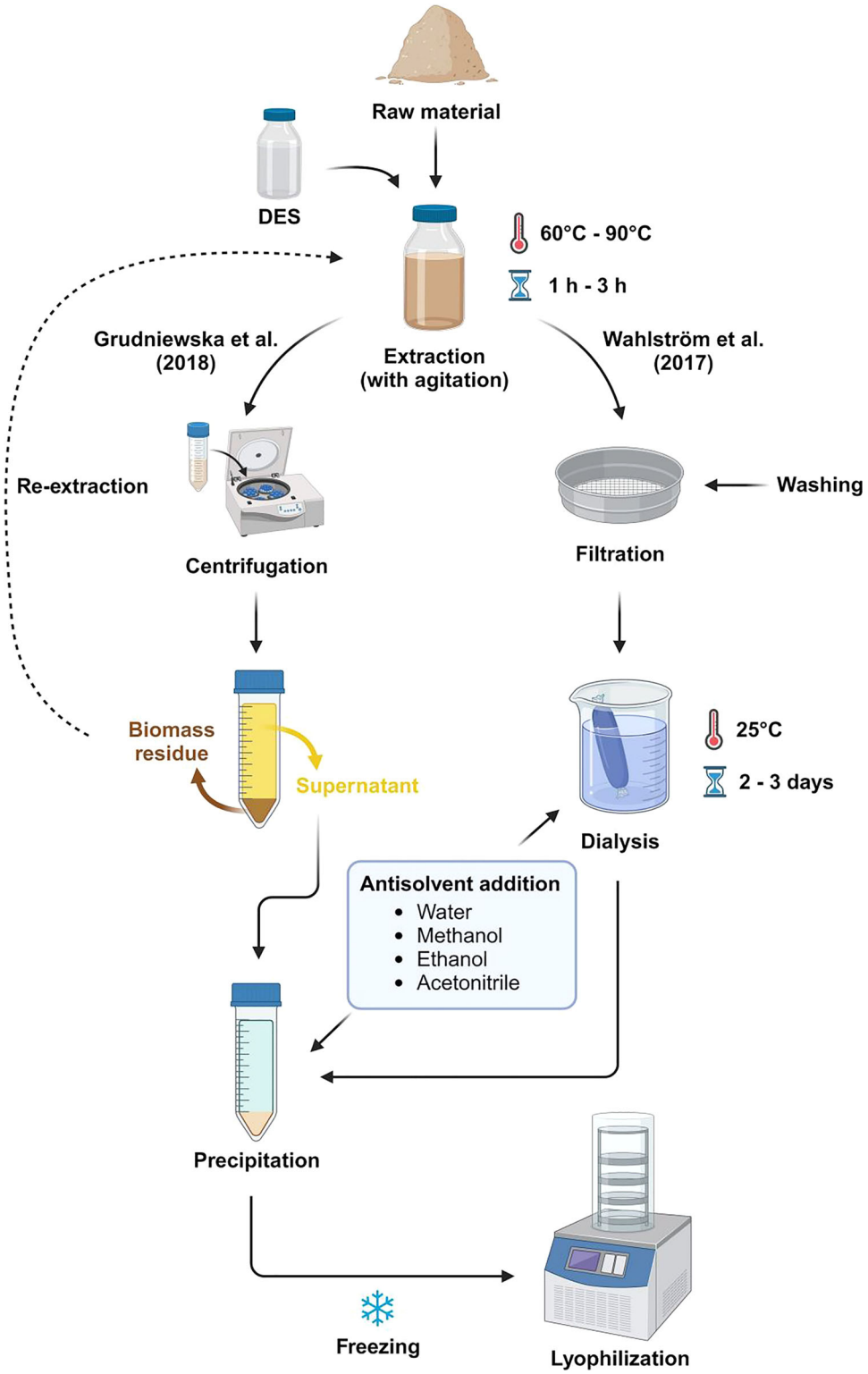

FIGURE 3 Two different procedures for deep eutectic solvent (DES)-mediated protein extraction from biomass. Antisolvent addition weakens the H-bonding between the hydrogen bonding acceptor (HBA) and hydrogen bonding donor (HBD), diluting the DES and leading to the loss of solubilization capacity.

DES-mediated extraction increased just marginally from 0.3192 to 0.3462 g when compared to the conventional alkaline extraction–acid precipitation method.

Yue et al. (2021) attempted to extract oat proteins by using butanediol isomers (1,2-butanediol, 1,4-butanediol, and 2,3-butanediol) as HBD in different molar ratios with ChCl as the HBA. Subsequently, ChCl–2,3-butanediol–H_2O (1:3:1) was shown to exhibit high extraction yield (10.69%–11.90%) and recovery rate (40.39%–42.92%) relative to other DESs tested in their study, whereas samples extracted by ChCl–1,4-butanediol–H_2O (1:3:1) had the highest protein content (55.82%). Additionally, proteins extracted by ChCl–1,4-butanediol–H_2O (1:3:1) had better solubility, foaming capacity, and stability (Yue et al., 2021). The ability of the DES to modify protein structure during the extraction process has been shown to be the cause of differences in the physicochemical and functional characteristics of the extracted proteins. In a subsequent study, Yue et al. (2022) examined the role of the hydrocarbon chain length of HBD (1,2-ethanediol, 1,3-propanediol, and 1,4-butanediol) with ChCl as HBA. Their study indicated that ChCl–1,3-propanediol–H_2O (1:2:1) had the highest protein content (62.50%) in samples, along with relatively high extraction yield and protein recovery yield (8.18% and 35.76%, respectively) compared to other DESs tested. The extraction mixture was centrifuged, and then the pellet was remixed with the same DES an additional three times. As noted previously, the additional DES treatment may have allowed higher concentrations of nonprotein constituents to be extracted, which might have lowered both the recovery yield and purity.

Gluten is a storage protein mostly derived from grain crops such as wheat and, to a limited extent, in rye, barley, and oats; it is composed of two components, gliadin and glutenin, which have a major role in contributing toward the formation of protein–protein interactions necessary for the structure and viscoelastic property of bread dough (Ooms & Delcour, 2019; Shewry, 2019). It possesses some features of structural proteins, including repetitive amino acid sequence regions and disulfide-bond cross-links among protein molecules, forming high molecular weight aggregates (Shewry, 2019).

According to the Osborne (1908) classification system, gliadins are prolamins, whereas glutenins are glutelins, comprising up to 30% and 50% of total proteins in wheat grains, respectively (Urade et al., 2018). The insolubility of gluten in aqueous solutions can make its extraction difficult for different reasons, including diagnostic assays for its detection or structure–function studies (Diaz-Amigo & Popping, 2013; Fallahbaghery et al., 2017; Ortolan et al., 2022). As demonstrated by Lores et al. (2017), utilizing a fructose–citric acid DES to solubilize gluten led to better extraction, and the process, when compared with a commercial kit for gluten detection, revealed nonsignificant changes in gluten concentration for samples of gluten-containing foods. The DES-extracted gluten fluorescence spectra showed a similar response as aqueous ethanol–extracted gluten, whereas the SDS–PAGE results showed a similar protein profile with slight differences, particularly at lower molecular weight for DES-extracted gluten. A similar study by Svigelj et al. (2017) highlighted the use of ChCl–ethylene glycol (1:2) and ChCl–urea (1:2) for gluten extraction, where both DESs extracted more gluten than the reference 60% ethanol–water solvent. It has been noted that matrix effects can alter the detection of gluten by enzyme-linked immunosorbent test (ELISA), which is an essential step in estimating the amount of gluten in foods (Xhaferaj et al., 2020). Aqueous ethanol solvent extraction can often be inadequate and usually necessitates the addition of denaturing reagents such as 2-mercaptoethanol and dithiothreitol (Doña et al., 2008; Segura et al., 2021). Without the use of additional reagents, the extraction of gluten by DESs is a simpler and safer approach.

Beyond the typical agricultural by-products produced from oilseeds and cereals, protein from nontraditional biomasses could potentially become accessible via DES-mediated extraction. Lin et al. (2021) utilized DESs to extract proteins from bamboo shoots and processing wastes and optimized the extraction parameters via response surface methodology (RSM). Their efforts revealed that ChCl–levulinic acid (1:6, containing 40% water v/v) optimally extracted 39.16 mg protein/g bamboo shoot (dry weight), corresponding to approximately 4% extraction yield. More pertinently, the extraction yield via DES is higher than the conventional NaOH-based method, which extracted 23.88 mg protein/g bamboo shoot (dry weight). When compared with other DES-mediated protein extraction schemes in Table 3, the extraction yield is substantially low. It is possible that ChCl–levulinic acid did not have the necessary protein solubilization capability. However, their study did not indicate whether they performed screening of DESs beforehand; thus, it is unclear whether or not ChCl–levulinic acid is suitable for protein extraction. There is need to evaluate the efficacy of some other DESs that could be more suited to extract bamboo proteins.

Fruit by-products are an emerging bioresource yet to be fully exploited for protein, and at the same time, they contain a considerable amount of high-value bioactive compounds beneficial for human health, including anthocyanins, carotenoids, and polyphenols, among others (Baker & Charlton, 2020; Jiménez-Moreno et al., 2019; Rodríguez García & Raghavan, 2021). The extraction of these bioactive compounds by DESs is the subject of an increasing number of reports. Nevertheless, there is a lack of knowledge regarding the co-extraction and recovery of proteins, and the bulk of research to date has only recorded the extraction of target compounds/molecules without taking co-extracting substances into account (Gullón et al., 2020; Vanda et al., 2018). It would be interesting to assess the diffusion or selectivity of DESs against various bioactive substances due to the variable solvation capacities of different DESs; this would help to determine which DES is better suitable for the extraction of particular compound. A study by Panić et al. (2021) proposed a biorefinery approach for the valorization of orange peel waste to extract D-limonene, polyphenols, and protein. Among several ChCl or sugar as HBA DESs containing either sugar or polyols as HBD, glucose–glycerol (1:2, 80% water w/w) followed by glucose–ethylene glycol (1:2, 50% water w/w) showed the highest protein extraction potential. Rico et al. (2021) employed sodium acetate:urea DES to evaluate its effectiveness for oligosaccharides and protein extraction from melon peels. They reported a maximum protein extraction yield of 39.91% by sodium acetate–urea–H_2O (1:3:1.6).

It is apparent that different DES compositions have been successfully used to extract proteins from various plant-based sources,

highlighting the flexibility and tunability of DESs as a solvent. Screening of the most effective protein-extracting DES is essential, as some DESs appear to extract proteins more effectively from certain biomass. Despite the different possible combinations of starting materials to synthesize DESs, ChCl–glycerol in particular was the most commonly used DES able to extract proteins efficiently. The exact mechanism that influences this process has yet to be fully explained.

Protein diffusion into DESs appears to be nonselective, but this also applies toward other compounds such as polysaccharides and polyphenols. Repeatedly treating the biomass with DES could lead to increased concentrations of nonprotein compounds, reducing protein purity. On the other hand, this could also become a method of choice by which one can modulate the degree of protein purity by simply increasing the number of times the sample is treated with the DES. As revealed by Jia et al. (2021) and Kornet et al. (2021), high-purity protein isolates are not necessary if the functionality of the lower purity concentrate fulfills its intended purpose within the food system. Nevertheless, obtaining high-purity protein isolates from DES-mediated processes requires further investigation. Future research can be directed to examine how to improve protein recovery yields and purity from plant-based sources by DES-mediated processes.

Given that conventional extraction methods placed emphasis on the types of proteins (i.e., albumins and globulins) made soluble by different solvents, it would be interesting to see how DES extracts different protein fractions in plant-based sources. The majority of research employing DESs as protein extraction solvents involves the use of nontraditional protein sources. The capacity of DES to solubilize the polysaccharide fraction, which makes up the majority of plant biomass composition, makes them appealing for enhanced utilization in the processing of biomass because this allows proteins to diffuse into the extracellular environment. Recalcitrant lignocellulosic materials pose the most challenging difficulty for processors to deal with among the several barriers to protein solubilization because of the enormous volume produced. Thus, valorizing lignocellulosic biomass for protein extraction is one of the more exciting possibilities for enhanced DES consumption. The range of source materials suitable for protein extraction has shown the value of DESs as innovative protein extraction solvents.

6.2 | Extraction of structural proteins using DESs from non-plant biomasses

The ability of DES to solubilize insoluble structural proteins, which are insoluble in common aqueous solvents, shows remarkable properties and capability of DES as powerful yet relatively benign solvents. Animal-derived biomasses are an especially rich source of these proteins. Structural proteins, according to a definition offered by Numata (2020), are proteins that possess a characteristic amino acid sequence or repeating sections and form the backbone or contribute to the mechanical properties of a living organism, cell, or material. Some examples of structural proteins include collagen, keratin, actin, and fibrin.

Collagen is an important structural element in animal tissue and is present in up to 30% of total proteins in vertebrates, being the primary constituent of extracellular matrices such as skin, bones, and ligaments (Subhan et al., 2020). Collagen is a fibrous protein composed of three polypeptide chains intertwined in a triple-helix configuration. These chains are rich in amino acids like glycine, proline, and hydroxyproline. These three amino acids together often form repeating segments that provide the unique structure of collagen (Shoulders & Raines, 2009). The repeating pattern of amino acids in each alpha chain helps form a tightly wound helical structure. Multiple triple-helix collagen molecules align side by side to create fibrils, which further associate into larger fibers. The strength and stability of collagen arise from the intermolecular forces between the chains and the precise arrangement of amino acids, leading to its essential role in providing structural support and integrity to various tissues in the body (Furtado et al., 2022).

Collagen has long been utilized as a highly functional biomaterial in food, cosmetics, and biomedicine, therefore garnering interest in its production (Sherman et al., 2015; Subhan et al., 2020). There has been great interest in exploring the extraction of collagen from non-mammalian species (particularly from fish and its by-products) due to religious considerations, food safety issues, and impetus to reduce waste generated from food processing (Gómez-Guillén et al., 2011). Batista et al. (2022) utilized a citric acid–xylitol–H_2O (1:1:10) DES to extract collagen from blue shark skin and obtained a 16.1% protein extraction yield, representing a 2.5-fold improvement over the conventional acid extraction approach along with a considerable reduction in processing time (1 h against 96 h). Their investigation also showed that the DES-extracted collagens are comparable to conventionally extracted collagen in terms of chemical composition and cytotoxicity (Batista et al., 2022). Their work highlights the use of a completely different type of DES compared to the typical ChCl-based DESs used to extract proteins.

On the other hand, Bai et al. (2017) reported the extraction of collagen peptides from cod skins using six different DESs, with the efficacy of DES in the order of ChCl–oxalic acid > ChCl–acetic acid > ChCl–lactic acid > ChCl–ethylene glycol > ChCl–glycerol > ChCl–urea. Analysis on the effect of HBA:HBD ratio revealed that ChCl–oxalic acid at a 1:1 ratio had optimal protein extraction efficiency and purity. Their study further showed that ChCl–urea was completely incapable of extracting any peptides or proteins, in contrast to studies by Wahlström et al. (2017) and Lin et al. (2022), which demonstrated that ChCl–urea had the ability to solubilize proteins. It should be noted, however, that these examples involve different kinds of proteins. The screening of DESs that may solubilize proteins is necessary due to the large range of combinations between different HBA and HBD at varied molar ratios. The choice of these DESs can differ based on the biomass and particular protein characteristics.

Keratin is derived from hair, wool, feathers, beaks, nails, hooves, and claws of animals (Feroz et al., 2020). Due to its high protein content, attempts have been made to extract keratin from those raw materials. However, the dissolution of keratin is difficult due to disulfide bonding between cysteine residues. The disulfide bonding provides structural

stability and rigidity, hindering solvent penetration (Jiang et al., 2018). Previous attempts to extract keratin used a variety of techniques ranging from thermal, chemical, and enzymatic processes (Giteru et al., 2023). Some of those techniques involve harsh chemicals and process conditions that may negatively affect keratin functionality. Out of all those methods, only sulfitolysis, oxidation, and enzymatic processes are potentially viable to generate edible keratin (Giteru et al., 2023).

Therefore, research is ongoing to identify more sustainable and efficient methods to produce edible keratin from keratin waste biomass. Nuutinen et al. (2019) showed that sodium acetate–urea (1:2) at 100°C for 6 h is capable of solubilizing up to 86% of poultry feathers, resulting in 45% keratin yield. Zhang et al. (2021) investigated the use of ChCl–oxalic acid to process poultry feathers. They found that ChCl–oxalic acid (1:2) with 30% (%wt) water, at 80°C for 6 h, produced 29.24% keratin yield and was best suited to extract chicken feather keratin, with a 91.31% dissolving rate. Zhang et al. (2023) developed a different set of DESs to extract the keratin from chicken feathers as a result of this research. They first experimented with two different DES types (ChCl–phosphoric acid and urea–phosphoric acid) at a number of different HBA:HBD ratios (1:3, 1:4, and 1:5). ChCl–phosphoric acid (1:5) had the highest keratin extraction yield as a result, at 82.02%. After that, they made an effort to formulate ternary DESs by adding $ZnCl_2$ to the aforementioned DESs. However, only a modest increase was achieved (up to 85.46% yield) by ChCl–phosphoric acid–$ZnCl_2$ (1:5:2). This was attributed to increased viscosity upon addition of $ZnCl_2$, which hindered solvent interaction with keratin. Apart from poultry feathers, wool keratin has also been considered another viable source to produce edible keratin. Similar DESs have been investigated in the extraction of wool keratin (ChCl–urea & ChCl–oxalic acid), as shown by Moore et al. (2016), Jiang et al. (2018), as well as Wang and Tang (2018). Shavandi et al. (2021) followed a different approach by using L-cysteine–lactic acid (2:20, w/v) at 95°C for 3.5 h. Their approach resulted in the dissolution of 90% of the material, although the keratin yield was not disclosed.

Okoro et al. (2022) made an effort to improve upon this. They claimed that when 0.5 g of wool were mixed with 1.6 g of L-cysteine in 20 mL of lactic acid (DES 1.6:20, w/v) and heated to 105°C for 8 h, 93.77% of keratin was produced. The capacity of various DESs to disrupt the intermolecular connections that hold the keratin fiber structure together, notably the disulfide bonds, may have varied, which could explain differences in protein extraction yield between various studies. Different processing conditions might have been linked to varying extraction yields of protein (Okoro et al., 2022).

When dealing with structural proteins such as collagen and keratin, acidic DESs were proven to be most effective for extracting these proteins due to their ability to break down cross-links in these proteins' fibrous structures. Acidic DESs facilitate the solubilization of collagen and keratin by cleaving the cross-links, thus disrupting the rigid fibrous structure and enabling the solvent to penetrate the protein interior (Bai et al., 2017; Shavandi et al., 2021). This contrasts with the DESs used to extract proteins in plant biomass, where ChCl–glycerol was the most often used DES. Plant proteins are often more soluble because of their globular structure, whereas proteins from animal-derived biomass form complex cross-linked fibrous structures. How DESs interact with the proteins is influenced by the proteins' structural features.

6.3 | Synergistic combinations of DESs with other extraction technologies

Other research studies have used additional processing technologies like ultrasound- and microwave-assisted procedures to further increase the yields of protein extracted using DESs as the solvent (Table 4). This includes work by Liu et al. (2017), where pumpkin seed was evaluated for protein extraction by ChCl–polyethylene glycol 200 (1:3). In their study, up to 93.95% protein was extracted following optimization of a sequential ultrasound–microwave synergistic method. This study was notable for two reasons. First, it was among the first to showcase the potential of combining DES-mediated extraction with synergistic treatments to increase protein extraction yield. Compared against other studies that did not employ synergistic treatments, Liu et al. (2017) achieved >90% protein extraction yield by using complementary processing methods. As shown in the study, synergistic treatments like these further improve the sustainability of the entire process due to the amount of protein extracted, the reduced water use, and the decreased extraction time. Second, this was one of the first studies to assess the effectiveness of protein recovery using various protein precipitation procedures following DES-mediated extraction. They experimented with four distinct protein precipitation methods.

The isoelectric precipitation method simply utilized pH adjustment to the overall protein p*I*. The ethanol precipitation method attempted to utilize the ability of ethanol to displace the hydration layer on the protein surface, destabilizing hydrogen bonds between protein molecules, thus promoting hydrophobic interactions leading to aggregation and precipitation. For the self-precipitation method, they simply added the original DES solution to induce precipitation by the volume exclusion principle. They found that the all-methods-combined technique was superior compared to the others. However, their study did not investigate deeper into the effects of the precipitation techniques on the protein structure and, subsequently, their functional properties.

Hernández-Corroto et al. (2020) extracted proteins from pomegranate peel by an ultrasonic-assisted process, with ChCl–acetic acid–H_2O (1:1:10) that extracted up to 15 mg protein/g peel. Optimization of processing parameters (including DES component molar ratios, ultrasound amplitude, and extraction time) yielded further increases up to 20 mg protein/g peel. Pomegranate peels contain about 3.3% protein, so DES extraction would provide roughly 46%–61% of the total protein in pomegranate peels (El Barnossi et al., 2021). The use of high-voltage electrical discharges (HVEDs) as a pretreatment to encourage cell rupture for greater release of proteins and polyphenols was examined in later research by Hernández-Corroto et al. (2022). In comparison to untreated samples, HVED-treated samples displayed increased protein and polyphenol diffusion.

Guzmán-Lorite et al. (2022) employed a similar strategy to extract proteins from pomegranate seeds, and they investigated protein

TABLE 4 Summary of studies that incorporated synergistic technologies to facilitate deep eutectic solvent (DES)-mediated protein extraction.

Protein source	DES (molar ratio of HBA to HBD)	Extraction parameters	Extraction yield	Protein content ($N \times 6.25$)	Reference
Wheat flour	Fructose–citric acid (1:1), 20% DES diluted in water (v/v)	*Ultrasound-assisted extraction* Time: 15 min Solid/Solvent ratio: 1:40 (w/v) Ultrasonic amplitude: 40%	95.4%	–	Lores et al. (2017)
Sacha inchi seed meal (*Plukenetia volubilis* L.)	ChCl–glycerol (1:2)	*Sequential ultrasound-assisted extraction* Time: 20 min Solid/Solvent ratio: 1:10 (w/v) Ultrasonic power: 40 W Ultrasonic amplitude: 50% Ultrasonic frequency: 20 kHz	38.17%	77.43%	Sharma et al. (2023)
Pumpkin seed (*Cucurbita moschata*)	ChCl–PEG 200 (1:3)	*Simultaneous microwave and ultrasound extraction* Temperature: 43°C Time: 4 min Solid/Solvent ratio: 1:28 (w/v) Microwave power: 120 W Ultrasonic power: 240 W	~95%	–	Liu et al. (2017)
Pomegranate peel (*Punica granatum* L.)	ChCl–acetic acid–H_2O (1:1:10)	*Ultrasound-assisted extraction* Time: 11 min Solid/Solvent ratio: 3:100 (w/v) Ultrasonic amplitude: 30%	20 mg/g peel	–	Hernández-Corroto et al. (2020)
Pomegranate seeds (*Punica granatum* L.)	ChCl–glucose (1:1), with 20% water (wt%)	*High-voltage electrical discharge (HVED) pretreatment* HVED energy: 160 kJ/kg Pulses: 300 Time: 11.5 min Solid/Liquid ratio: 1:9 (w/v) *Extraction* 20% DES added to pretreated mixture Temperature: 50°C Time: 1 h Stirring: 160 rpm	4 g/100 g Seed	–	Hernández-Corroto et al. (2022)
	ChCl–acetic acid (1:2), with 35% water (v/v)	*Ultrasound-assisted extraction* Temperature: 60°C Time: 15 min Amount of sample: 27.8 mg Ultrasonic amplitude: 80%	52.8%	–	Guzmán-Lorite et al. (2022)
		Successive ultrasound (in DES)-pressurized liquid extraction (in alkaline buffer)	94.4%	–	
Wheat germ	Potassium carbonate–glycerol (1:3)	*Microwave-assisted extraction* DES concentration: 0.52 g/mL Time: 3 min 28 s Solid/Solvent ratio: 1:39 (w/v) Microwave power: 186 W	33%	–	Olalere and Gan (2023)

Abbreviations: ChCl, choline chloride; HBA, hydrogen bonding acceptor; HBD, hydrogen bonding donor.

extraction yields from a singular method or successive extractions by combining different techniques. Successive extraction initially by ultrasound-assisted DES followed by pressurized liquid extraction (PLE) extracted up to 23.8 g protein/100 g seed, representing 94.4% of pomegranate seed protein. Here, complementary extraction methods combined together extracted more proteins than singular methods. Ultrasound processing can disrupt cell walls, leading to greater release of proteins into the extracellular environment, whereas PLE promotes greater cell wall disruption and solvent diffusion, as well as perturbation of protein structure as a result of high temperature and pressure, thus unfolding the protein and exposing more surface area to the solvent and increasing solubility (Zhou et al., 2021). In summary, syn-

ergistic processing techniques may allow increased protein extraction yields where stand-alone DES use is insufficient.

Aqueous biphasic systems or aqueous two-phase systems (ATPSs) employ liquid–liquid partitioning to separate molecules such as proteins by exploiting the incompatibility between two different polymer-aqueous solutions mixed together (Asenjo & Andrews, 2011). The target molecules, in this case being proteins, will migrate toward the solution (phase) it has an affinity with. Zeng et al. (2014) first showed the application of DES-based ATPS (DES-ATPS) by introducing 2 mL of 0.6 g/mL K_2HPO_4 to 1.4 g of ChCl–urea (1:2) and then adding 10 mg of protein (bovine serum albumin, BSA). After mixing at room temperature, BSA was present in the DES phase at 99% extraction yield, suggesting that DES-ATPS can be an efficient method to fractionate proteins. Xu et al. (2015) studied the protein-partitioning behavior in DES-ATPS comprising K_2HPO_4 added to ChCl–ethylene glycol (1:2), ChCl–glycerol (1:1), ChCl–glucose (2:1), or ChCl–sorbitol (1:1). Under optimized conditions, 2 mL of 0.9 g/mL K_2HPO_4 added to 1.3 g of ChCl–glycerol (1:1) followed by mixing 10 mg of BSA at 30°C resulted in 98.16% extraction of BSA into the DES phase. This study showed that BSA kept its conformation because its circular dichroism (CD) and UV–vis spectra were identical before and after extraction.

Li et al. (2016) then used betaine as HBA to synthesize a series of DESs to develop a DES-ATPS to extract BSA. Phase-forming ability with the salt solution is key to the applicability of DES-ATPS; thus, betaine–urea–H_2O (1:2:1) was selected for further study on the basis of their phase-forming ability and BSA extractability. Under optimum conditions, betaine–urea–H_2O (1:2:1)/K_2HPO_4 extracted up to 99.82% BSA. Up until then, only DES/salt ATPS mixtures had been investigated as a means to fractionate proteins. Meng et al. (2019) showed that DES/DES ATPS is a viable method to extract proteins with high efficiency. They found that tetrabutylammonium chloride–polypropylene glycol 400/L-proline–xylitol (TBAC–PPG400/pro–xyl) performed the best among several combinations of amino acids–polyol DESs.

These studies provide further evidence that DESs can extract proteins, albeit with varying efficacy. In the study by Li et al. (2016), betaine-based DESs can extract BSA effectively (>~60% extraction efficiency), whereas trypsin and ovalbumin were separately extracted to a lesser degree. Most DES-ATPSs were screened on the basis of its efficacy to extract BSA and not the other proteins in a particular study. Xu et al. (2015) also observed that DESs do not have selectivity of specific proteins that are mixed together, which is beneficial for food protein extraction as it would support maximal protein recovery from biomass. The basis for the interaction between DESs and proteins has not yet been formally established, except for model proteins. We shall discuss this further in the next section. Overall, DES-ATPS seems to be a particularly effective method to fractionate some proteins with high efficacy. However, studies that examine the performance of DES-ATPS extraction on waste or by-product biomass are lacking.

The majority of published research had focused on extraction of model proteins, and therefore, the performance of DES-ATPS systems on biomass is yet to be fully assessed. Nevertheless, we envision that DES-ATPS could be a useful method to further purify high-value protein fractions after the initial extraction process to solubilize proteins and then to be purified by ATPS afterward. Marchel et al. (2020) demonstrated the use of betaineHCl-based DESs to extract pepsin from porcine gastric mucosa powder and obtained extraction efficacies of 96.6%–98.8% from the DESs tested. When compared with conventional protein extraction methods, ATPS-based methods may generate undesirable effluents, thus making them costly for scale-up. Recovery of the phase constituents can make the process more economical but would require further study. So far, no studies have been made with regards to the recycling of DESs and their protein extraction efficacy after recycling. Panić et al. (2019) have examined this idea. They extracted the anthocyanin with ChCl–citric acid and then made an effort to recover both the anthocyanin and the DES. Their method got 96.8% DES recovery and 99.46% anthocyanin recovery. Such studies might improve the overall cost-effectiveness and sustainability of DES-mediated protein extraction.

7 | MECHANISM OF DES-MEDIATED PROTEIN SOLUBILIZATION AND EXTRACTION

As an emerging extraction technology, DES-mediated protein extraction is currently in its early stages and requires further research to elucidate extraction mechanisms. These investigations hold the potential to optimize extraction yields, determine the suitable HBA and HBD composition, and reveal any potential alterations to protein structure resulting from DES–protein interactions. To aid in understanding the DES-mediated extraction mechanism, it can be useful to compare it with a conventional food protein extraction process as outlined by Preece et al. (2017). As the proteins in lignocellulosic biomass tend to be confined within cells and are therefore not immediately exposed to the solvent, we focused more on the general method of extraction based on this material. Because they are not bound by a cell, protein-rich animal-derived biomass is frequently solvent-accessible; as a result, the solubilization technique is covered separately.

The general steps of DES-mediated protein extraction for lignocellulosic biomass closely follow conventional methods, with differences arising from solvent-specific effects. Cell disruption of biomass is necessary for protein extraction. The effect of particle size reduction on the efficacy of protein extraction has been documented, whereby particle size reduction of soybeans may improve protein extraction, due to increased surface area available for the solvent to permeate through (Russin et al., 2007; Vishwanathan et al., 2011). However, Rommi et al. (2015) showed that particle size reduction of rapeseed press cake did not influence protein recovery. Their investigation revealed that extensive milling had a negative impact on protein solubilization due to denaturation. Although some milling is required to rupture otherwise rigid cell walls and facilitate protein release, excessive milling should be avoided, as research has shown diminishing benefits of such intense pretreatment. Meanwhile, some DESs have been shown to be able to dissolve the lignocellulosic barrier of biomass. To what extent substantial particle size reduction might be advantageous for protein recovered by DES is still unknown. Nevertheless, it is believed that pro-

tein extraction from biomass by DESs is improved as a result of some DESs' capacity to disrupt lignocellulosic structure (Mankar et al., 2021).

Once the solvent is in contact with proteins, protein-solvent interactions determine the overall mass transfer rate with a higher mass-to-solvent ratio, increasing the amount of protein solubilization (Kamal et al., 2021) (Figure 2d). In alkaline extraction, protein solubilization is influenced by solvent parameters, such as pH, ionic strength, temperature, extraction time, and solid/solvent ratio. The solvent pH plays an important role in overall protein solubilization by altering the protein surface charge distribution. The resulting electrostatic attraction toward water and repulsion between proteins promotes protein solubilization, whereas the addition of salts introduces ionic species that can mask opposing charges on the protein surface, impacting protein–protein and protein–water interactions (Li & Xiong, 2021).

In general, proteins exhibit a net positive charge at pH levels below their p*I* and gain a net negative charge at pH levels above their p*I*. Protein solubility increases as the pH moves away from the p*I*. Proteins from the majority of plant-based raw materials have p*I* ranging from pH 4 to pH 5 (Day et al., 2022; Grossmann & McClements, 2023), and extraction typically occurring at pH > 8 allows the proteins to acquire a net negative charge, thus inducing electrostatic interactions with solvent molecules and thereby promoting solubilization (Momen et al., 2021). This has been the basis for protein extraction from food sources up till today.

DES-mediated protein extraction does not necessarily rely on the typical pH-influenced electrostatic attraction/repulsion as a result of changes in the ionization state of amino acid residues favored by alkaline solvent extraction for protein solubilization. Instead, it seems to be based on extensive hydrogen-bond networks by DESs that stabilize protein structure (Bubalo et al., 2023). A variety of DESs with different pH levels appear to be able to solubilize proteins, indicating that pH is not the main driver of protein solubilization with DESs.

There is a distinction between DESs that can dissolve insoluble proteins from plant biomass and DESs that can dissolve complex and fibrous proteins from animal-derived biomass. For instance, ChCl–glycerol excels in solubilizing proteins derived from plant-based biomasses. Protein structure can be protected, and pH-induced protein denaturation can be avoided by using DESs with a neutral pH. Additionally, in the neutral pH range, polymerization with polyphenolic species can be avoided, thus preventing excessive browning associated with exposure to alkaline pH levels, as well as preventing alterations in protein solubility and other functional properties (Czubinski & Dwiecki, 2017). Many of the proteins characterized from plant biomass are globular in nature, such as legumins and vicilins. Their shape and structure are due to the exposure minimization of hydrophobic amino acid regions to the typically aqueous solvent environment.

The remarkable solubilization capability of DESs for on proteins, especially insoluble proteins, has yet to be investigated and explained in detail. Given that individual components of typical DESs can be classified as osmolytes, DESs could act similarly as osmolytes to stabilize the protein structure in solution. Osmoytes are small organic molecules that regulate and protect cells from osmotic shock (Bolen, 2004). This phenomenon has been shown in studies where proteins were exposed to a highly concentrated environment with osmolytes (Rabbani & Choi, 2018). Choi et al. (2011) proposed that NADES naturally occur within plant cells and act as a medium participating in the biosynthesis of phytochemicals, biocatalysis, and desiccation protection, which protect the plant from environmental extremes. However, they did not elaborate on the similarity between the role of NADES and osmolytes in protein stabilization, although the study by Zeng et al. (2016) seems to add evidence for the link between osmolytes and DESs by showing that a betaine–urea DES can counteract protein denaturation, given that both betaine and urea are commonly identified as osmolytes.

Recently, Bubalo et al. (2023) proposed that DES may indeed act as osmolytes. The study revealed that a range of novel NADESs can be formed by combining a few frequently encountered osmolytes. Their study expanded on the range of DESs compatible with proteins. The synthesized DESs exhibited the ability to stabilize a model protein. The ability of DESs to solubilize previously insoluble proteins could be due to DESs exerting an osmolyte-like effect, inducing denatured proteins to refold to a soluble configuration due to the "crowding" effect of DESs (Maity et al., 2020) (Figure 2e).

Meanwhile, the dissolution of keratin provided an insight on how DESs solubilize insoluble fibrous proteins. The main factor influencing this is the ability to disrupt the extensive keratin protein–protein cross-links facilitated by disulfide bonding. According to Shavandi et al. (2021), carboxylic acid–based DESs can form hydrogen bonds with the various functional groups in wool keratin, promoting fiber swelling. This leads to solvent penetration throughout the protein molecule. The acidic environment then facilitates hydrolysis of the peptide bonds, producing lower molecular weight fragments, as shown by Wang and Tang (2018), yielding soluble keratin. DES-mediated collagen extraction follows a similar pathway. Acidic DESs can destabilize the cross-links that maintain the triple-helix structure, dissociating the individual polypeptide chains (Jafari et al., 2020).

Furthermore, the acids can depolymerize collagen into lower molecular weight fragments, making them more soluble and thus increasing extraction efficacy. Bai et al. (2017) demonstrated the use of ChCl–oxalic acid (1:1) to extract collagen. They proposed that the formation of ammonium salt, hydrogen ions, and imino in collagen was driving the extraction of collagen peptides from cod skins. However, collagen depolymerization into peptides can be controlled by performing the extraction procedure at lower temperatures. Batista et al. (2022) and Bisht et al. (2021) employed lower extraction temperatures compared to Bai et al. (2017) and achieved extracts displaying the distinctive α1, α2, and β chain bands in SDS–PAGE. These bands resembled those of collagen extracted through conventional methods, implying the preservation of DES-extracted collagen's structure.

Overall, a few combinations of HBA and HBD have been shown to be capable of protein solubilization with different extraction mechanisms. To date, model proteins such as BSA and lysozyme have been used to study the effect of DESs on protein solubility, conformation, and stability, whereas similar studies on food-derived proteins are lacking (Xin et al., 2017). The protein extraction efficiency of DESs is governed by the set of physicochemical properties inherent to each DES mixture, in conjunction with the characteristics of the protein (i.e., amino acid

composition and conformation). These physicochemical properties include the melting point, density, viscosity, polarity, and hydrophilicity/hydrophobicity of the DES mixture. The interdependency between the various physicochemical properties of DESs and protein extraction efficacy requires further attention. Additionally, processing conditions have an impact on the physicochemical characteristics of DES, which in turn affects protein solubilization. Therefore, understanding the factors influencing DES-mediated protein extraction is crucial for expanding its food-related applications.

7.1 | Factors influencing DES-mediated protein extraction

Based on Table 3, DES-mediated protein extraction efficiency is subject to a few processing conditions, such as type of DES, water content in DES, extraction temperature and time, solid-to-solvent ratio, and agitation rate. To establish an ideal condition where protein extraction will be maximized, these factors can be varied. Currently, one-factor-at-a-time and RSMs are employed to optimize extraction conditions (Chen et al., 2021; Hewage et al., 2024).

The distinct physicochemical characteristics of DES may have a variety of effects on protein extraction. Although there are numerous HBA:HBD combinations that can solubilize proteins, it is currently unclear how all of those properties interact to affect protein extraction. In order to maximize the extraction of biomolecules from plant sources, new strategies incorporating computer-aided technology, such as machine learning, are very helpful when analyzing complicated data with various parameters (Ma et al., 2023; Shekhar et al., 2023). Shi et al. (2022) successfully employed a machine learning algorithm to predict DES viscosity by considering the basic properties (i.e., molar mass of HBA and HBD, HBA:HBD molar ratio, and temperature), molecular fingerprint of the HBA:HBD composition, and water content as the model input. Thus, machine learning can potentially accelerate efforts to understand the inter-relationships between the various factors involved in DES-mediated protein extraction.

7.1.1 | Type of DES

The selection of DES varies according to the nature of biomass selected. As can be noted from Table 3, plant-based proteins are best extracted using polyol-based DESs, whereas animal-derived proteins can be better solubilized by acidic DESs. Different types of DES will have different protein extraction capabilities, so it is essential to screen for the most effective DESs. Once a suitable DES is selected, the HBA:HBD ratio needs to be adjusted as it plays an important role in protein extraction efficacy. Changes in the HBA:HBD ratio will alter the solvent's physicochemical characteristics, which will alter its ability to extract proteins. According to Chen et al. (2021), increasing the molar amount of glycerol from 1 to 3 while keeping the molar amount of ChCl at 1 results in an increase in extraction yield, but increasing the glycerol molar amount to 4 results in a drop in yield.

7.1.2 | Physicochemical properties of DESs

Each DES type, at different HBA:HBD ratios, exhibits different physicochemical properties. These properties include polarity, pH, viscosity, density, and conductivity (Hansen et al., 2021; Omar & Sadeghi, 2022). Each property may affect protein extraction yield differently. The physicochemical properties of DESs have been widely studied, but so far no studies have been conducted to find correlations between the physicochemical properties of DESs with protein extraction efficacy. Such correlations may be helpful in fully comprehending how the solvent interacts with the protein, which may make it easier to select the best DES for protein extraction.

7.1.3 | Water content in DESs

Given that water content and DESs' physicochemical characteristics are closely related, DESs' water content is a significant component. Addition of water disrupts the intermolecular hydrogen bonding between the HBA and HBD, reduces the viscosity and density of DESs, and increases its polarity (Chen, Yu, et al., 2019; Töpfer et al., 2022). Most DESs possess high viscosity, potentially reducing processing efficiencies. Therefore, the addition of water is a practical method of reducing DES viscosity. However, the addition of water may affect protein extraction yield, as shown by Chen et al. (2021). In this case, the balance between high protein extraction efficacy and solvent viscosity needs to be considered.

7.1.4 | Solid-to-solvent ratio

The solid-to-solvent ratio, expressing the proportion between solid biomass and DES, is commonly measured in terms of weight per volume or weight per weight basis. Optimal selection of this ratio enables the design of a balanced process, optimizing both protein extraction yield and process efficiency. Lower ratios enhance extraction by increasing the solvent volume, expanding the surface area for protein diffusion. On the other hand, increasing the ratio (e.g., from 1:10 to 1:5) elevates the biomass proportion, resulting in increased protein extraction yields owing to the higher total protein content available for extraction. This drives mass transfer toward the protein-deficient solvent. However, there exists an optimum ratio for different biomass samples; further increases in ratio may not enhance extraction yields due to saturation of available solvent surface area for protein binding.

7.1.5 | Extraction temperature and time

At higher temperatures, the increased kinetic energy that molecules gain promotes greater solvent mobility, which translates into lower viscosity. The combination of reduced solvent viscosity and heightened kinetic energy enhances interactions between solutes and the solvent,

thereby increasing solubility. During DES-mediated protein extraction, optimum temperature ranging from 60 to 90°C was observed to be effective for the optimum recovery of proteins from various biomasses (Table 3). High temperatures, though, which are higher than the temperature at which proteins begin to denature, could cause denatured proteins to clump together and form insoluble aggregates. For instance, extraction from rapeseed cake and evening primrose seed cake at higher temperatures (140°C) was demonstrated to give higher levels of protein-rich precipitates, but they also developed dark colors due to nonenzymatic browning at high temperatures (Grudniewska et al., 2018).

Extraction duration is also a key parameter in extraction processes. Typically, extraction yields increase over time up to a certain point, as protein diffusion into the solvent is influenced by factors, such as solvent viscosity, extraction temperature, and agitation rate. Prolonging extraction time beyond the optimum may bring about protein degradation, depending on the temperature and DES type, as seen with acid-based DESs (Bai et al., 2017). The complexity of the biomass structure and composition further shapes the optimal extraction time. In the context of plant-based biomass, proteins enclosed within cells require an initial disruption of the cell wall for solvent penetration and subsequent protein solubilization. The degree of cell wall disruption and protein molecular size determine the efficacy of protein transport from the cell interior, with cell wall structure potentially hindering the process. Depending on the biomass, extraction times reported in the literature range from 45 min to 18 h, with the majority requiring 1–2 h for efficient extraction (Table 3). However, synergistic processing methods, such as ultrasound-, microwave-, and high pressure-assisted processing, can be used to speed up the extraction process, reducing extraction time to 4–15 min (Table 4).

7.1.6 | Agitation rate

Agitation of the extraction system is designed to facilitate the diffusion and solubilization of protein to the solvent, enhancing extraction efficacy. Agitation can be induced by mechanical stirring or ultrasonic cavitation. As noted previously, the high viscosity of pure DESs may require vigorous agitation to help proteins solubilize into DESs. Although the majority of studies have reported agitation, stirring, or shaking as part of the extraction process, only a few investigated the effect of agitation rate on extraction efficacy. Chen et al. (2021) observed that an increase in stirring speed enhanced soybean protein extraction yield, using ChCl–glycerol. They found that an agitation rate of 873 rpm by mechanical stirring was optimal.

7.2 | Recovery of DES-solubilized proteins

Recovery of proteins solubilized into DESs is a critical process in the entire extraction scheme due to multiple factors such as the nature and structure of protein, the composition of the solvent, and process conditions. Bowen et al. (2022), Hewage et al. (2022), and Zhou et al. (2022) revealed that protein recovery or its back-extraction from DESs is a limiting factor toward the wider adoption of DESs as extraction media. Conventional methods exploit the physicochemical properties of proteins, such as their solubility, charge, and hydrophobicity, to selectively precipitate proteins out of the solvent. Manipulating the pH, ionic strength, and temperature allows us to selectively precipitate and recover proteins from the solvent. The same strategies could also be used to recover proteins after DES-mediated extraction.

Adjusting the pH to the p*I* of a protein, a technique known as p*I* precipitation, is commonly used for protein recovery (Contreras et al., 2019). At the p*I*, proteins carry zero net charge, where electrostatic repulsion is at its lowest, thus promoting their close association. Although highly effective for precipitating proteins with p*I* values within a narrow range, this method may be less suitable for proteins with significantly different p*I* values. Proteins with diverse p*I* values may not precipitate efficiently under the same pH conditions, potentially limiting the applicability of p*I* precipitation across a broader range of proteins. Alternatively, the manipulation of ionic strength by the addition of salts also serves to modulate the electrostatic potential at the protein surface by charge screening (Grossman & McClements, 2023). At low concentrations, salts enhance protein solubility by shielding charged groups on the protein surface, reducing electrostatic repulsion between proteins, and enabling more favorable interactions with solvent molecules. This occurs as ions in the solution form an ionic atmosphere around the protein, effectively screening its surface charge. As salt concentration increases, there is a threshold level where additional ions start to more significantly "screen" the surface charge on proteins. This diminishes electrostatic repulsion, promoting protein aggregation and precipitation. Further increase in salt concentration tends to reduce protein aggregation as protein–protein interactions are screened by ionic species that extend further into the solvent phase, thus increasing protein solubility (Li & Xiong, 2021). The reduced solubility at intermediate salt concentrations is often leveraged for protein purification. However, subsequent purification steps are typically necessary to remove excess salts and obtain a protein product suitable for downstream applications (Hansen et al., 2022). Finally, a mild heating step can be used to separate the soluble protein fractions from the high-molecular weight aggregates. This method is commonly used in leaf protein extraction to separate the white (soluble proteins) and green (chlorophyll and insoluble proteins) fractions (Møller et al., 2021). Care must be exercised to avoid increasing the temperature above the protein denaturation temperature, which leads to extensive protein aggregation and degrades technofunctional properties (Pérez-Vila et al., 2022).

Optimal precipitation methods following DES-mediated protein extraction from biomass have not yet been extensively studied. Liu et al. (2017) investigated precipitation methods by comparing among DES self-precipitation, p*I*, ethanol, and combinatory p*I*/ethanol/DES self-precipitation methods, resulting in 61.52%, 77.93%, 92.26%, and 97.97% precipitation efficiencies, respectively. Bai et al. (2017) compared the precipitation capability of methanol, ethanol, acetonitrile, and acetone. Acetone exhibited poor miscibility with all DESs they tested and was therefore unable to precipitate proteins. Acetonitrile

were miscible with all but two of the DESs tested, whereas methanol and ethanol was miscible with all DESs. Solvents miscible with proteins in DESs resulted in observable white flocculations, which were assumed to be proteins. UV–vis spectra analysis revealed that the solvents can have selective precipitation capability. Methanol and ethanol can precipitate collagen to a high degree of purity in ChCl–lactic acid, ChCl–acetic acid, and ChCl–oxalic acid, whereas ChCl–ethylene glycol and ChCl–glycerol precipitates presented higher amounts of impurities. Nevertheless, it remains unclear whether organic solvents are suitable for larger scale processes and presents environmental concerns that hinder such applications. Additionally, the impact of different recovery methods on the functional properties and nutritional quality of protein is yet to be fully determined.

Other studies, however, simply used water as an antisolvent, which offered varying protein extraction yields (Grudniewska et al., 2018; Wahlström et al., 2017; Yue et al., 2021). The difference in the yield could be because of the varying composition of proteins in the extract. Proteins that are normally insoluble in aqueous solvents would be susceptible to this technique. It could be that the DES–protein interactions that previously stabilized structure and conformation would be lost as the DES loses its characteristics when water content exceeds a certain amount, typically >50% (v/v), therefore resulting in a loss of solubility (Dai et al., 2015).

At this stage, there is also the possibility of incorporating DES recycling at the end of the extraction process to further improve the viability and sustainability of DES-mediated protein extraction. In this context, DES recycling involves the separation of DES from the protein extract and subsequent recovery of DES for successive extractions. Isci and Kaltschmitt (2021) reviewed different DES recycling techniques available. The most suitable technique should ideally be amenable to a number of criteria, including DES recovery efficacy, energy requirements, process simplicity, and cost-effectiveness (Isci & Kaltschmitt, 2021). DES recycling by antisolvent addition has been shown to retain DES extraction capability after a few rounds of recycling, as seen in the extraction of rutin from tartary buckwheat hull (Huang et al., 2017). Water was used as an antisolvent to precipitate rutin, and the water was evaporated to recover the ChCl–glycerol DES. They reported the extraction efficacy reduced from 92% to 81% after three rounds of DES recycling.

However, it is unclear whether complete recovery of DESs is possible or otherwise. In other words, the recycled DES may contain impurities coming from the biomass. This is because antisolvent addition also precipitates other types of solutes (Isci & Kaltschmitt, 2021). The effect of recycled DES on protein extraction efficacy is yet to be demonstrated. Moreover, protein recovery, combined with other DES recovery methods, represents an underexplored area of research deserving further investigation. Overall, there is a need for research in this field because protein recovery yields are crucial for figuring out whether the extraction process and source material are practically viable for use in commercial applications.

8 | THE INFLUENCE OF DESS ON THE STRUCTURAL, TECHNOFUNCTIONAL, AND NUTRITIONAL PROPERTIES OF PROTEINS

So far, DES-mediated protein extraction has been shown to have some desirable properties when compared with conventional alkaline extraction. A comparison of selected attributes between DES-mediated protein extraction and conventional alkaline extraction is shown in Table 5. However, the impact of DESs on proteins extends beyond its solubilization capabilities. Solvents in contact with proteins can affect protein structural configuration, which further influences both the functional and nutritional properties of the recovered protein (Bader et al., 2011; Kim et al., 2021). In high pH environments (pH > 10), denaturation and structural alterations occurring could modify protein functional properties (Deleu, Lambrecht et al., 2019). Ruiz et al. (2016) demonstrated through SDS–PAGE that the extraction of quinoa proteins by aqueous NaOH at pH 11 resulted in a decreased abundance of higher molecular weight bands when compared to samples extracted at pH 8–10. Those high molecular weight proteins may have undergone denaturation and subsequent peptide bond hydrolysis catalyzed by high pH, induced formation of smaller protein fragments. Organic solvents, on the other hand, can denature proteins by inducing protein tertiary structure to become disordered due to the weakening of the hydrophobic effect stabilizing it (Magsumov et al., 2020; Pace et al., 2011). DESs by nature are nonaqueous and yet can solubilize proteins. Because of this, the direct impact of DESs on protein structure and configuration is of great interest because understanding the impact of DES–protein interactions can help us modify the parameters of the extraction process to produce functional proteins.

8.1 | Changes in protein structure

It is important to understand how protein structure changes as a result of DES-mediated protein extraction. Zeng et al. (2014) found that BSA in ChCl–urea (1:2) had similar UV–vis spectra to those in pure water, indicating that the BSA conformation was preserved. Another study analyzed the structures of BSA and lysozyme in ChCl–glycerol (1:2) water mixtures (50% and 75% DES by weight) and phosphate buffer using CD and small-angle neutron scattering approaches (Sanchez-Fernandez et al., 2017). Proteins in aqueous DES and buffer were shown to possess similar conformation, whereas pure ChCl–glycerol (1:2) led to partial unfolding. Partial unfolding would suggest that lysozyme function would be affected. However, a follow-up study showed that lysozyme stored in ChCl–glycerol (1:2) for 40 days at room temperature retained activity after dialysis and subsequent rehydration in aqueous buffer (Sanchez-Fernandez et al., 2022). This adds to the report from Esquembre et al. (2013) showing temperature cycling from 25°C–80°C–25°C resulted in lysozyme refolding in aqueous buffer, partial refolding in ChCl–glycerol, and irreversible unfolding

TABLE 5 Comparison between deep eutectic solvent (DES)-mediated protein extraction and conventional alkaline protein extraction with regard to selected attributes.

Attribute	DES-mediated extraction	Alkaline extraction
Protein solubilization	Solubilizes a wide range of proteins, including plant- and animal-based proteins	Solubilizes mainly plant-based proteins
Protein denaturation	Using DESs compatible with milder conditions can reduce protein denaturation	High pH can lead to protein denaturation, affecting functional properties and nutritional quality
Solvent toxicity	Generally considered safer and more environmentally friendly since many DESs are derived from natural components and are nontoxic	May be hazardous due to the use of caustic chemicals (e.g., NaOH) at high concentrations
Sustainability	Typically considered more sustainable due to the use of biodegradable and renewable solvents, and potentially reduced energy use	May have a higher environmental impact due to chemical usage

in ChCl–urea. Zeng et al. (2016) showed that lysozyme was unfolded and then regained its tertiary structure after being exposed to a 25°C–80°C–25°C heating and cooling cycle in hydrated (25%, 50%, and 75% wt) betaine–urea (1:2).

However, Sanchez-Fernandez et al. (2017) showed that BSA exposed to heat at 80°C in a pure ChCl–glycerol environment and then re-equilibrated at 25°C remained denatured and did not regain its original conformation as measured by CD. They noted that the presence of water facilitated BSA folding, and that the partially unfolded state of BSA in pure ChCl–glycerol reflects the change in solvent–protein interactions. Based on these results, it appears that some proteins are able to refold in the presence of DES, with the presence of some hydration and the type of DES also being an important factor to consider. In other words, generalizations of DES–protein interactions must be made carefully and should consider the DES type, composition (i.e., HBA:HBD composition), hydration level, and the identity of the protein. The ability of DESs to impart structural and thermal stability to proteins was highlighted by Yadav et al. (2020), showing sustained α-chymotrypsin activity in both ChCl–urea and ChCl–glycerol. Sarkar et al. (2017) performed molecular dynamics simulations and showed that ChCl and urea in a 1:2 ratio did not denature the chicken villin headpiece subdomain protein (HP-36). Their simulations revealed that increased concentrations of ChCl antagonized the effects of urea-mediated HP-36 denaturation in two ways. First, hydrogen bonding occurs between ChCl and urea, which results in the formation of a DES, preventing urea from binding to the protein surface. Second, hydrogen bonding between the DES and the protein backbone stabilized its structure and prevented HP-36 from being denatured and preserving its solubility. These studies provide important insights into the influence of DESs on protein structure and conformation.

Some studies on food-derived proteins have shown that the secondary structures of DES-extracted proteins are slightly different from those of alkaline-extracted proteins (Yue et al., 2021; Yue et al., 2022; Lin et al., 2022). It is yet unclear how specific plant proteins such as soy glycinin and β-conglycinin, pea vicilin, or legumins would behave when exposed to DESs, and the interactions between DESs and proteins that are water-insoluble are yet to be fully established. As mentioned in Section 7.1, DESs could likely act similarly as osmolytes that interact closely at the protein surface, conferring structural stability. Oat proteins extracted by DES had high denaturation temperatures, ranging from 105 to 111°C as measured by differential scanning calorimetry, indicating that DES-mediated extraction retained the structural integrity of oat proteins (Yue et al., 2021). Similarly, DES-extracted soy proteins had higher thermal stability than alkaline-extracted proteins (Chen et al., 2021). The majority of research that has examined DES–protein interactions to date has focused on distinct, globular water-soluble proteins, which allowed for incredibly thorough in silico characterization work. It is yet unknown how certain biomass-derived protein types alter structurally when exposed to DES systems and whether DESs have an impact on these proteins' functional characteristics.

For structural proteins such as collagen and keratin, DES treatment appears to facilitate deconstruction of the main protein structure, without significant changes to its secondary structure. Wang and Tang (2018), through X-ray diffraction (XRD) analysis, showed the α-helix structure of wool was destroyed by ChCl–oxalic acid (1:2) during the dissolution process. Furthermore, SDS–PAGE results indicated that protein fractions were between 3.3 and 7.8 kDa, due to the hydrolysis of keratin. Jiang et al. (2018) reported that ChCl–urea (1:2) treatment of wool resulted in a hierarchical dissolution of keratin layers, leading to solvent penetration toward the inner layers of keratin. Interestingly, XRD spectra showed that raw wool and DES-treated wool exhibited a similar pattern, showing two distinctive peaks, indicating that the α-helix structure was retained to some degree. Clearly, acidic DESs such as ChCl–oxalic acid are much more aggressive toward keratin than other DESs as the strong acidity of the DES promotes hydrolysis of peptide bonds (Bai et al., 2017). However, Shavandi et al. (2021) showed that the use of L-cysteine–lactic acid (2:20 w/v) produced a keratin extract with the characteristic α-helix XRD spectra peak along with an increased proportion of β-sheet structure.

8.2 | Changes in protein technofunctional properties

The functionality of proteins is significantly influenced by their structure, making any alteration capable of modifying its functional properties. However, there is a lack of studies aimed at characterizing the impact of DES-mediated protein extraction on its functional properties. Yue et al. (2021) compared the foaming capacity and stability of oat proteins extracted by ChCl-based DESs with butanediol isomers as HBD. Their study showed that butanediol isomers and added water content have some influence on oat protein foaming properties, which are related to thermal stability and slight alterations to the secondary structure of oat proteins. When 1,4-butanediol was used as the HBD without the addition of water, the extracted proteins had the lowest denaturation temperature. However, the addition of water (ChCl–1,4-butanediol–H_2O at 1:3:1 ratio) increased the denaturation temperature of the extracted oat proteins. The foaming capacity mirrored this trend with the addition of water resulting in higher foaming capacity. This effect was explained by the addition of water to the DES, which modified the polarity of the extraction environment, thus altering the secondary structure of oat proteins.

Yue et al. (2022) expanded on this by examining the role of the hydrocarbon chain length of dihydric alcohol as HBD, and addition of water to DES on the functional properties of extracted oat proteins. The primary and secondary structures of oat proteins are mostly preserved by DES extraction, but some changes occur depending on the type of DES. Increasing the hydrocarbon chain length led to an increase in protein denaturation temperatures. Environmental pH affects the solubility, foaming, and emulsification properties of oat proteins more than the type of DES, influencing the eventual application to a food system. It was found that oat proteins extracted by hydrated DESs had better functionalities than those extracted by anhydrous DESs at the same pH level. Overall, their study provided insight into the effect of tuning DES composition on protein functional properties. Another study by Olalere and Gan (2023) extracted defatted wheat germ proteins using a synergistic microwave-DES approach and obtained protein concentrates with enhanced functional properties compared with a conventional alkaline extraction method. However, it is unclear whether the synergistic treatment or DES alone had a greater influence on the functional properties of protein concentrate.

8.3 | Changes in protein nutritional quality

Existing studies have primarily concentrated on identifying appropriate DES systems for various biomass sources and assessing the extraction efficacy, physicochemical, and functional properties of the extracted protein. However, there is still a knowledge gap regarding the potential impact of DES-mediated protein extraction on protein quality. The extent to which DES-mediated protein extraction can influence changes in protein quality remains uncertain. Only a few studies have explored the effect of DES-mediated extraction on protein quality. Seabuckthorn seed meal protein was extracted using several DES systems, including ChCl–glycerol (1:2), ChCl–urea (1:2), and ChCl–oxalic acid (1:1). The amino acid profiles of these DES-extracted proteins exhibited minimal differences when compared to proteins extracted through the conventional alkaline method. Furthermore, the essential-to-nonessential amino acid ratio for DES-extracted proteins (ranging from 0.40 to 0.45) either matched or slightly exceeded (0.40) that of alkaline-extracted proteins (Lin et al., 2022). This suggests that DESs do not chemically alter the amino acid composition, thus maintaining protein quality. One of the concerns related to the use of alkaline extraction methods is the potential for unintended degradation of protein quality at high pH levels (Jiang et al., 2009).

Proteins may undergo intramolecular or intermolecular cross-linking at high pH as a result of unfolding, exposing thiol groups on cysteine residues to form disulfide bonds (McKerchar et al., 2019). Therefore, protein aggregates may form, and these aggregates may become more resistant to digestion, thus reducing digestibility and amino acid bioavailability. By utilizing neutral-pH DESs for extraction below denaturation temperatures, proteins can retain their native configurations, leading to concentrates that are less susceptible to aggregation and with improved digestibility. However, DESs also have the capacity to extract nonprotein substances, including carbohydrates and phenolic compounds, which could potentially interact with the extracted proteins. Interactions between polyphenols and proteins can result in reduced protein solubility (Malik & Saini, 2017). Consequently, there is a need to identify whether co-extraction of other compounds along with protein yields a net positive benefit or the opposite. In the era of optimal nutrition, proteins are assessed for both their nutritional value and their nutraceutical qualities, which have the potential to have a favorable impact on physiological well-being.

9 | NUTRACEUTICAL POTENTIAL OF DES-EXTRACTED PROTEIN CONCENTRATES/ISOLATES

DESs have a remarkable capacity to extract a wide range of biomolecules with the potential to improve human health. Due to their special characteristics, DESs are excellent media for the valorization of raw materials, which results in a production of functional food ingredients. Functional foods—foods or dietary components that have favorable physiological benefits beyond merely supplying nutrients—owe their healthful effects to bioactive substances, often known as nutraceuticals (Granato et al., 2017). Proteins, peptides, polysaccharides, pre- and probiotics, polyunsaturated fatty acids, and other phytochemicals like polyphenols and carotenoids are only a few examples of the wide variety of these bioactive molecules (Granato et al., 2020). Guzmán-Lorite et al. (2022) provided evidence that DES-extracted proteins can act as substrates for the production of bioactive peptides. Guzmán-Lorite et al. (2022) successfully used ultrasound-assisted extraction of pomegranate seeds with DES as the solvent in their investigation. Notably, proteins extracted using DES showed better antioxidant activity than proteins extracted using alkaline techniques, which could be attributed to the DES's superior ability to extract pro-

teins. However, it is important to recognize that the co-extraction of phenolic compounds may also have an impact on the antioxidant activity. DESs have been shown to be efficient at extracting nonprotein components with potential for bioactivity, such as polysaccharides and polyphenols (Luo et al., 2023; Redha, 2021).

Future assessments of the bioactivity of DES-extracted protein concentrates and isolates, especially those from plant sources, should take into account the co-extraction of different chemicals due to the high solubilization capacity of DESs. However, the safety of the extracted protein products/extracts needs to be assessed.

10 | SAFETY CONSIDERATIONS FOR DES UTILIZATION IN FOOD PROTEIN PROCESSING

The cost-effectiveness and safety of the procedures used must both be considered when employing new methods. According to Benvenutti et al. (2019), interest in DESs has been sparked for a variety of applications, particularly those involving food, due to its potential to be a less toxic substitute for conventional organic solvents and ILs from the perspectives of both human exposure and environmental impact. Studies pertaining to DES biodegradability and toxicity are ongoing, given the enormous number of possible DES combinations. Afonso et al. (2023) compared the ecotoxicology effect among DESs, ILs, and organic solvents in terms of EC_{50} or the half-maximal effective concentration of the solvents against the bioluminescence of *Vibrio fischeri*, and they concluded that ILs are more ecotoxic than organic solvents and DESs. Although researchers have claimed DESs as safe and nontoxic solvents, we must evaluate their safety profile within the context of their application (Ferreira et al., 2022). A study by Li et al. (2023) highlighted the toxicity of amino acid–based DESs when compared to ChCl-based DESs. This is similar to the study by Juneidi et al. (2015) that shows several ChCl-based DESs (i.e., ChCl–glycerol, ChCl–urea, and ChCl–ethylene glycol) are less toxic than some cholinium salt–based DESs and ILs. The toxicity of amino acid–based DESs in the study by Li et al. (2023) was attributed to the high acidity of the HBA:HBD composition. This emphasizes the importance of cautiously choosing HBA:HBD combinations for safer protein extraction. The concentrations of DES constituents remaining in protein concentrates and their possible toxicity are unclear. Proteins extracted in this manner could potentially be contaminated with DES. However, the risk of DES contamination can be alleviated by dialysis of the protein concentrates. Currently, the majority of DES shown to be effective in protein extraction are limited to a few types; thus, future efforts should focus on establishing the safety and toxicity profile of protein concentrates obtained from DES extraction.

The solubilization capacity of DESs highlights their versatility as extraction solvents. However, the co-extraction of antinutritional factors (ANFs) with proteins can be problematic, as ANF limits nutrient bioavailability, digestion, and absorption, potentially leading to nutritional deficiencies (Samtiya et al., 2020). ANF encompasses different classes of compounds, including tannins, phytate or phytic acid, saponins, oxalates or oxalic acid, glucosinolates, anti-vitamin factors, gossypol, lectins, protease inhibitors, and amylase inhibitors (Kadam et al., 2021). Biomass valorization utilizes by-products that typically contain high ANF concentrations (Mattila et al., 2018; Nehmeh et al., 2022). The protein extraction method can play a role in determining the extent of ANF present in the final product (Amin et al., 2022). Previous studies have shown that wet extraction methods are more effective in minimizing the presence of ANF in protein isolates than dry fractionation (Amin et al., 2022). If a DES system used for protein extraction can co-extract significantly high concentrations of ANF, it could reduce the nutritional quality of the protein produced. More studies are needed to investigate the types of ANF and their quantities co-extracted with proteins by DESs and determine appropriate strategies to mitigate their negative influence on protein quality.

One of the advantages of DES-mediated protein extraction is that some DES systems can be used under relatively milder conditions (i.e., at lower temperatures or relatively neutral pH). Extraction at high pH has been associated with the formation of LAL (Deleu et al., 2019; Gerrard, 2002; Hou et al., 2018). Generally, under highly alkaline conditions, lysine residues react with dehydroalanine (DHA) intermediates formed through the loss of a proton from the α-carbon from serine, cysteine, phosphoserine, and cystine residues to form LAL (McKerchar et al., 2019). The reaction involves the nucleophilic addition of the lysine amino group to the electrophilic carbon atom in the DHA double bond, resulting in the formation of LAL and a modified lysine residue containing a DHA moiety (Friedman, 1999). The formation of LAL is generally considered undesirable because it can affect the nutritional quality and safety of the affected proteins, and steps are often taken to minimize its formation during food processing (Li, Zhang, et al., 2021). Given that some DES compositions have been shown to extract proteins under mild conditions, LAL formation could be minimized through a DES-mediated extraction process.

The allergenic potential of DES-extracted proteins is another aspect yet to be fully explored. Some food proteins possess high allergenic potential due to their biomolecular properties that promote their binding to IgE antibodies, thus provoking an immune response (Costa et al., 2020). Introducing novel proteins from the valorization of biomass could also expose previously unknown allergens. The allergenic potential of food proteins can be reduced, enhanced, or even left unchanged by different processing methods, which are mostly related to conformational changes undergone by the proteins in such a way that epitopes may no longer bind with antibodies (Rahaman et al., 2016). The extent of protein conformational change affected by DES is yet to be fully understood, and none of the existing studies have addressed the allergenic aspect of DES-extracted proteins. As discussed in Section 8.1, DESs have minimal effects on protein structure and conformation. It is possible that epitopes remain intact in protein concentrates. Thermal treatment is a common method to inactivate heat-labile proteins and concurrently disrupt conformational epitopes, thus reducing allergenicity, with the type of heat applied (dry vs. wet) also expressing different allergenic effects (Rahaman et al., 2016). The increasing prevalence and adoption of nonthermal processing methods, in conjunction with DES-mediated extraction, could alter protein conformation and reduce allergenicity. DES-extracted proteins could

be subject to regulations, such as the EU novel food law requiring substantial food safety evaluation (Verhoeckx et al., 2016). More studies are needed to assess the potential and risk of DES-extracted proteins to induce allergies.

11 | TECHNO-ECONOMIC ASSESSMENT OF DES-MEDIATED PROTEIN EXTRACTION

As novel solvents, DESs require extensive evaluations of their feasibility before considerations are made to deploy them on a wider scale. Investigators employ techno-economic assessment (TEA) to analyze cost/benefits of the proposed process and then determine its future direction in terms of research and investments (Quinn & Davis, 2015). TEA is a methodology used to evaluate the economic feasibility of various processes, by incorporating process design and simulation, accounting of mass and energy balance, estimation of the economic indicators (i.e., operating expenses, capital expenses, revenue generated, internal rate of return, payback period, and net present value, among others), and sensitivity analysis to evaluate how changes in price/cost of some parameters would impact the feasibility of the project (Fu et al., 2023). Kumar et al. (2020) highlighted that the economic sustainability of DES-mediated processing of lignocellulosic bioresources is dependent on generating high-value, multiproduct sidestreams. Other studies reported the TEA of DES-based biorefinery processes such as the production of fermentable sugars (Huang et al., 2020) and bioethanol (Peng et al., 2021) from rice straw. Another study looked into the TEA of integrated DES/IL-mediated pretreatment of corn stover for 5-hydroxymethylfurfural and lignin production (Zhao et al., 2022).

Despite increasing interest in DES-mediated food protein extraction, no studies have been reported on TEA of DES-mediated protein extraction processes. TEA should be conducted for different bioresources along with the DES used, revealing whether protein extraction will add value to the valorization of the bioresources or otherwise. Cost of raw materials taken from underutilized bioresources is expected to be competitive compared against conventional protein sources. However, this may be subject to limitations in the supply chain and cultivation conditions. For example, the production scales for different bioresources vary considerably, and some may depend on seasonality, as seen in some crops (Roni et al., 2023). In another example, biomass yield produced varied from year-to-year as a result of climate and irrigation practices that affected production (Muneer et al., 2021).

Variations in HBA:HBD composition can impact the cost of the proposed process, particularly when certain combinations lead to extreme pH levels, as observed in acidic DESs, necessitating the use of higher cost anticorrosive vessel materials (Ahmed et al., 2021). Following the multiproduct approach recommended by Kumar et al. (2020), DES-mediated bioresource valorization is anticipated to yield greater value and sustainability compared to conventional processes. However, a sensitivity analysis by Peng et al. (2021) highlighted that the selling price of ethanol generated from DES-mediated pretreatment of rice straw can be influenced by fluctuations in ChCl prices and other parameters. As operations scale up, the availability of DES components may be subject to market pressures, impacting costs. Implementing DES recycling could enhance the cost-effectiveness of the process. In conclusion, further research is essential to address the critical gap in determining the economic feasibility of DES-mediated food protein extraction processes.

12 | CONCLUSION AND FUTURE PERSPECTIVES

As we approach the year 2050, it becomes increasingly evident that traditional food processing methods must undergo reevaluation to meet the expected surge in demand. We must develop innovative processes that align with sustainability goals. DESs represent a novel frontier in food protein extraction, classified as environment-friendly solvents with numerous advantageous characteristics that could simultaneously satisfy sustainability objectives and consumer demands. However, like many emerging processes, the scalability and feasibility of this extraction process on a larger scale need to be demonstrated. Given the various combinations of HBA and HBD, it is imperative to screen for DESs capable of effectively solubilizing proteins. Expanding the range of HBA and HBD combinations with favorable protein extraction capabilities broadens our solvent options and could potentially help avoid bottlenecks associated with limited supply. Moreover, generalizations about DES–protein interactions must be approached with caution due to the diverse HBA:HBD compositions. For example, polyol-based DESs tend to solubilize proteins by enveloping and stabilizing them against aggregation, whereas acidic-based DESs often disrupt inter-protein bonds and hydrolyze peptide bonds, leading to smaller molecular weight fragments. The tunability of DES properties through composition adjustments requires further optimization, and the feasibility will also depend on the supply chains for their individual components.

Currently, the majority of studies on DES-mediated protein extraction have primarily focused on extraction conditions and yield. To gain a comprehensive understanding of DES-mediated protein extraction, it is imperative to explore the influence of DES composition on the structure and conformation of the extracted proteins, including the impact of specific HBA or HBD on extraction yield. Increasing the extraction yield also requires a closer look at the protein recovery process, whereby further optimization is needed. Based on our literature review, DESs may act similarly to osmolytes that could stabilize proteins against aggregation, acting as a molecular "crowder," forcing unfolded proteins to adopt a compact configuration. Meanwhile, the assessment of changes in protein functional properties, nutritional quality, and nutraceutical capacity remains relatively underexplored. Any changes in these aspects are likely attributable to modifications occurring during the extraction process, allowing us to select the most suitable DES for producing protein concentrates with the desired functionality and quality. Meanwhile, some in vitro and in vivo bioassays must be used to evaluate the nutraceutical potential in addition to assessing the nutritional value of DES-derived protein extracts/concentrates and associated hydrolysates. By using computer

simulation approaches like molecular docking, the structure–activity relationship of such protein hydrolysates and biopeptides needs to be determined.

Furthermore, the potential toxicity, allergenicity, and the presence of ANF in DES-extracted proteins need to be addressed. Given the solubilization capacity of DESs, we must consider the co-extraction of other compounds alongside proteins concerning both functionality and product safety. Co-extraction of nonprotein fractions can lower protein concentrate purity, so future research can investigate methods to enhance protein purity following DES-mediated protein extraction. It is essential to note that novel food regulations in many regions worldwide demand rigorous safety assessments before ingredients and foods produced using novel methods can gain approval for use. Therefore, researchers must evaluate these safety risks to enhance the scope of DES-mediated protein extraction over a wide range of nutra-pharmaceutical applications.

AUTHOR CONTRIBUTIONS

Conceptualization; investigation; visualization; writing—original draft: Mohamad Ariff Hanafi. *Writing—review and editing*: Farooq Anwar. *Supervision; funding; writing—review and editing*: Nazamid Saari.

ACKNOWLEDGMENTS

This work was supported by Universiti Putra Malaysia, under the Geran Putra Berimpak scheme (Project No.: GP-GPB/2022/9711600). All figures are made on BioRender.com.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known conflicts of interest or personal relationships that could have appeared to influence the work reported in this paper.

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How to cite this article: Hanafi, M. A., Anwar, F., & Saari, N. (2024). Valorization of biomass for food protein via deep eutectic solvent extraction: Understanding the extraction mechanism and impact on protein structure and properties. *Food Frontiers*, *5*, 1265–1301. https://doi.org/10.1002/fft2.389